THE IMMUNOPATHOLOGY OF ERYTHEMA NODOSUM LEPROSUM

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Declaration

I, Edessa Negera Gobena, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

Leprosy is a disease caused by *Mycobacterium leprae*, an acid-fast bacillus whose clinical spectrum correlates with the host immune response. Erythema nodosum leprosum (ENL) is an immune-mediated inflammatory complication causing high morbidity in affected leprosy patients. A case-control follow-up study was conducted in Ethiopia to test the hypothesis that ENL is associated with impaired immune regulation. In 46 patients with ENL and 31 lepromatous leprosy (LL) matched controls, the frequency of regulatory T-cells, memory T-cells and B-cells were analysed by flow cytometry. The *in vitro* pro-inflammatory cytokines production by peripheral blood mononuclear cells (PBMCs) to the response of *M. leprae* whole cell sonicate stimulation was determined by Enzyme-linked immunosorbent assay. The gene expression of these cytokines in the blood and skin biopsies was determined by quantitative polymerase chain reaction (qPCR) before and after treatment. Patients with ENL had lower percentage of $CD4^+$ regulatory T-cells than LL controls at recruitment. The percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells expressing activated T-cells were significantly higher in the PBMCs of patients with ENL than in LL controls before treatment. The *in vitro* production and gene expression of the cytokines: TNF-α, IFN-γ, IL-1β, IL-6, IL-8 and IL-17A were significantly increased in untreated patients with ENL. ENL patients had a higher median percentage of tissue-like memory (TLM) and activated memory (AM) B-cells than LL controls before treatment while the median percentage of total B-cells and resting memory (RM) B-cells did not significantly different in both groups before treatment. The level of anti-PGL-1, LAM and Ag85 antibodies were not significantly different in patients with ENL before treatment. Patients with ENL had significantly lower circulating C1q than LL controls before treatment. However, after treatment, the amount of circulating C1q was not significantly different in both groups. Our findings suggest that ENL is associated with reduced percentage of regulatory T-cells and increased $CD4^+/CD8^+$ T-cell ratio and this immune imbalance may lead to the initiation of ENL reactions in either permitting productions of antibodies critical to an immune-complex formation or as a cell-mediated immune response in patients with leprosy. Consequently, this study illuminates the role of T-cell activation in the pathogenesis of ENL reaction and challenges the long-standing dogma of immune-complexes as the sole aetiology of ENL reactions.

For my family

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ABBREVIATIONS

A	Adenine
AAERC	AHRI/ALERT Ethics Review Committee
Ab	Antibody
ACTH	Adrenocorticotropic Hormone
AFB	Acid-fast bacilli
AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immune Deficiency Disease
ALERT	All Africa Leprosy, Tuberculosis and Rehabilitation Training Centre
AM	Activated B-cells
AP	Alternative pathway
APC	Allophycocyanin
APC	Antigen Presenting Cells
BANDS	Bangladesh Acute Nerve Damage Study
BB	Mid Borderline
BD	Becton Dickinson
BI	Bacillary Index
BL	Borderline Lepromatous
Br	Relative Optical Background
BT	Borderline Tuberculoid
C	Cytosine
C1q	Complement C1-q
CD10	Cluster of Differentiation 10
CD127	Cluster of Differentiation 127
CD161	Cluster of Differentiation 161
CD19	Cluster of Differentiation 19
CD21	Cluster of Differentiation 21

CD25	Cluster of Differentiation 25
CD27	Cluster of Differentiation 27
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
CD45RO	Cluster of differentiation 45RO
CD62L	Cluster of differentiation 62L
CD8	Cluster of Differentiation 8
CIC	Circulating Immune Complex
CMI	Cell Mediated Immunity
CP	Classical Pathway
CR	Complement Receptor
CRP	C-reactive protein
CS	Complement System
CSE	Central Statistics of Ethiopia
CT	Threshold
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic Cell
DM	Diabetes Mellitus
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked ImmunoSpot
ENL	Erythema Nodosum Leprosum
ENLIST	ENL International STudy group
FACS	Fluorescence-activated Cell Sorting
FBS	Foetal bovine serum
FITC	Fluorescein Isothiocyanate
FMO	Fluorescence Minus One

FMOH	Federal Ministry of Health
FoxP3	Forkhead box P3
FSc	Forward Scattered
G	Guanine
G1D	Grade-1 Disability
G2D	Grade-2 Disability
G-CSF	Granulocyte Colony Stimulating Factor
GLP	Good Laboratory Practice
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
H & E	Hematoxylin and eosin
HDL	High desnsity Lipoprotein
HIV	Human Immunodeficiency Virus
HL	Hodges–Lehmann
HLA	Human Leukocyte Antigen
HRP	Horseradish Peroxidase
HuPo	Human acidic ribosomal Protein
IBD	Inflammatory Bowel Disease
IC	Immune-complex
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-10	Interleukin-10
IL-17A	Interleukin-17A
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
ILEP	International Federation of Anti-Leprosy Associations
INFIR	ILEP Nerve Function Impairment and Reaction
INOs	Inducible nitric oxides

iNOS	Inducible Nitric Oxide Synthase
KD	Kilo Dalton
LAM	Lipoarabinomannan
LDL	Low density Lipoprotein
LL	Lepromatous Leprosy
LSHTM	London School of Hygiene and Tropical Medicine
LST	Lymphocyte Stimulation Test
MAC	Membrane -Associated Complex
MB	Multibacillary
MBLP	Mannose-Binding Lectin Pathway
MDT	Multi Drug Therapy
MFI	Median Fluorescent Intensity
MHC	Major Histocompatibility Complex
MLWCS	*M.leprae* Whole Cell Sonicate
mRNA	messenger RNA
NCB	Naïve B-cells
NCBI	National Centre for Biotechnology Information
NCD	New Case Detection
ND-O-BSA	Natural Disaccharide Octyl Bovine Serum albumin
NO	Nitric Oxide
NRERC	National Research Ethics Review Committee
OCT	Optimum Cutting Temperature
OD	Optical Density
PAMPS	Pathogen-associated Molecular patterns
PAT	Platelet aggregation Test
PB	Paucibacillary
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis

PCP-1	Monocyte Chemotactic Protein-1
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cyanine-7
PEG	Polyethylene Glycol
PerCp-Cy5.5	Peridinin-chlorophyll proteins- Cyanine-5.5
PFA	Paraformaldehyde
PGL-1	Phenolic Glycolipid-1
PHA	Phytohaemagglutinin
PMNs	Polymorphonuclear Leukocytes
PMT	Photomultiplier Tubes
PNL	Pure Neuritis Leprosy
PRRs	Pattern Recognition receptors
PTA	Platelet Aggregation Test
qPCR	quantitative PCR
QR	Relative detection Efficiency
QST	Quality Control Test
RA	Rheumatoid Arthritis
RCB	Resting Memory B-cells
rCV	robust Coefficient of Variation
R-J	Ridley-Jopling
RMC	Red Medical Clinic
RNA	Ribonucleic acid
ROC	Receiver Operating Characteristic
RORC	RAR-related orphan receptor C
RP	Registered Prevalence
rpm	Revolution per minute
RPMI	Roswell Park Memorial Institute medium
RR	Reversal Reaction
rSDEN	Robust Standard Deviation of Electronic noise

RT	Room Temperature
SE	Standard Error of the mean
SLE	Systemic Lupus Erythematosus
SNPs	Single Nucleotide Polymorphisms
SSc	Side Scattered
SSS	Slit-Skin Smear
ST	Sensory Testing
T	Thymine
T1R	Type 1 Reaction
TB	Tuberculosis
TC	Total Cholesterol
TCM	Central Memory T-cells
TEC	Effector T-cells
TEM	Effector Memory T-cells
TG	Triglycerides
TGF-β	Tumour Growth Factor beta
TLM	Tissue-Like Memory B-cells
TLR	Toll Like Receptor
TMB	Tetramethylbenzidine
TNC	Naive T-cells
TNF-α	Tumour Necrosis Factor alpha
Tregs	Regulatory T-cells
TT	Tuberculoid
VDR	Vitamin-D Receptor
VEGF	Vascular Endothelial Growth Factors
VMT	Voluntary Muscle Testing
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1. Statement of the problem

Leprosy is a disease caused by *Mycobacterium leprae*, an intracellular acid-fast bacillus. It mainly infects the skin and peripheral nerves. Leprosy is a spectral disease forming a five-point spectrum with the localized tuberculoid leprosy (TT) and the generalized lepromatous leprosy (LL) forming the two poles of the spectrum. The clinical spectrum of leprosy correlates with the host immune response.

Leprosy reactions (Reversal reactions and Erythema Nodosum Leprosum) are immune-mediated inflammatory complications of the disease which can occur before, during or after successful completion of multi-drug treatment. They are a major cause of nerve damage and morbidity in a significant proportion of leprosy patients. Erythema Nodosum Leprosum (ENL) is an inflammatory complications of leprosy, manifesting as tender erythematous skin lesions and systemic features of disease often fever, neuriti s and bone pain.

Oral prednisolone is the drug of choice for ENL treatment but more than 40% of cases do not show clinical improvement. High recurrent episodes and flare-ups are common in these patients. Hence, treatment and management of reactional and post reactional episodes of ENL often pose a therapeutic challenge to leprologists.

The underlying immunologic mechanisms for ENL remain unclear with several different hypotheses. The hypothesis of ENL as an immune-complex mediated disease proposed in the 1960s has yet to be convincingly demonstrated. The involvement of cell mediated immunity in the pathogenesis of ENL has since been postulated. The phenotypic and functional roles of these cell-mediated immunity (T-cells) are not yet adequately investigated in leprosy reactions particularly in patients with ENL reactions. There are conflicting reports from various studies of ENL reactions regarding T-cell subpopulations and immune-complex deposition in the lesions. However, although these studies draw our attention to the importance of T-cells and immune-complexes in the evolution and subsequent course of ENL reactions, specific conclusion is not derived as yet. Most information on ENL

reactions are derived from routine clinical treatment of the disease and comprehensive well-designed studies with large sample size are lacking.

The difficulties in treating ENL reactions illustrate the importance of understanding the mechanisms of the disease. Since the cause(s) and mechanisms of reactions are poorly understood, treatment is often suboptimal. Therefore, understanding the immunopathogenesis of ENL reaction is important to improve diagnosis and treatment.

1.2. Research hypothesis

Erythema nodosum leprosum reaction is associated with increased percentage of activated T-cells due to decreased frequency of regulatory T-cells.

1.3. Aims of the study

The aims of the study were:
To describe the relative percentages and phenotypes of T-cells in patients with ENL before and after treatment compared to lepromatous leprosy patient controls.

1.4. Specific objectives

The specific objectives of the study were:

1. To investigate T- cell phenotypes (regulatory and memory T-cells) in patients with ENL compared to lepromatous leprosy patient controls
2. To define B- cell phenotypes in patients with ENL compared to lepromatous leprosy patient controls
3. To describe the kinetics of pro-inflammatory and regulatory cytokines production (IL-1β, TNF-α,TGF-β, IFN-γ, IL-6, IL-8 and IL-17) in patients with ENL and LL controls before and after treatment
4. To determine the level of gene expressions of pro-inflammatory cytokines /chemokines (IL-1β, TNF-α,.TGF-β, IFN-γ, IL-6, IL-8 and IL-17) in the blood and skin lesions of patients with ENL and LL controls before and after treatment
5. To determine the level of circulating C1q in the plasma of patients with ENL and LL controls before and after treatment

6. To determine the level of C1q gene expression in the blood and skin lesions of patients ENL and LL controls before and after treatment.
7. To determine the levels of anti ND-O-BSA (PGL-1), Ag85 protein and LAM antibodies in the plasma samples of patients with ENL and LL controls before and after treatment.
8. To describe the histological characteristics of ENL and LL lesions before and after treatment.
9. To describe the correlation between clinical and immunological profiles of ENL reactions.

1.5. Outline of the thesis

This thesis addresses the immunopathology of ENL. A case- control follow-up study was set-up to investigate the immune profiles of patients with ENL before and after treatment. Several immunological factors were investigated in the blood as well as in the skin lesions of patients with ENL reactions (cases) and patients with non-reactional LL (controls).

Chapter 1 outlines the rationale for carrying out this research. Chapter 2, presents the literature review of leprosy. This chapter gives the general introduction on leprosy, global and regional prevalence of the disease, diagnosis, treatment, management of leprosy complications and immunological profiles of each clinical type in the leprosy spectrum. The role of innate and adaptive immunity in each clinical forms of the spectrum has been reviewed and presented in this chapter.

Chapter 3 covers with the leprosy reactions. Types of leprosy reactions, the occurrence of leprosy reactions in leprosy spectrum, epidemiology of leprosy reactions (incidence and prevalence), risk factors, pathology, clinical pictures, diagnosis and treatment of leprosy reactions are described in this chapter. The challenges of diagnosis, treatment and management of leprosy reactions are also highlighted in the chapter. Chapter 4 describes gaps of the immunology of leprosy reactions. Current information on the role of innate immunity (complements, cytokines and neutrophils), adaptive immunity such as effector and memory T cells, regulatory T- cells and memory B-cells in the immunopathogenesis of ENL reactions are extensively reviewed in this chapter. Our current knowledge of the

underlying causes of leprosy reactions particularly ENL reactions is also reviewed and presented in this chapter.

Chapter 5 describes the materials and methods used in this study. It describes the study area, study design, case definitions and strategies used for patient recruitment and follow-up. Procedures and processes for the ethical clearance, consenting and compensating patients for their lost time are explained. Standard operating procedures for sample collection and subsequent processing are described. Detailed procedures for the assessment of various pro-inflammatory cytokines, T-cells, B-cells and complement C1-q in the blood as well as in the skin lesions are described. The outcome and explanatory variables of the study are defined. The data analysis approach and statistical parameters used in each sub-group analysis is justified. Finally, data interpretation and presentation methods are highlighted in this chapter. Chapter 6 comprises the findings of the study. It has 9 sections and the contents of each section is summarized as follow as:

Section-1: In this section the sociodemographic and clinical characteristics of study subjects are described. Study participants age profile, sex proportion, the MDT status of each patients at recruitment, HIV screening, bacillary index at leprosy diagnosis, the morphology of skin lesions in ENL, anatomical location of pain in ENL, histopathological features of ENL and LL skin lesions and the lipid profiles of cases and controls at enrolment are described in this section.

Section-2: This section presents the frequency of regulatory T- cells in the PBMCs of patients with ENL and LL controls before, during and after treatment. The relative frequency of $CD4^+$ and $CD8^+$ T-cells in the two groups are presented. The kinetics of regulatory T-cells before and after prednisolone treatment of patients with ENL is covered in this section.

Section-3**:** Section-3 accounts memory and T-cell activation in patients with ENL and LL controls before, during and after treatment. The relative frequency of naïve T- cells, effector T-cells, central and activated memory T-cells in the PBMCs of patients with ENL and LL controls are presented in this section. Changes in the frequency of activated and memory T-cells before and after prednisolone treatment of patients with ENL are summarized in this section.

Section-4: In this section the percentages of regulatory and memory T-cells in acute and chronic ENL are presented.

Section-5: This section presents the frequency of the sub-population of B-cells (resting memory B-cells, activated memory B-cells, tissue like memory B-cells and naive B-cells) in patients with ENL and LL controls before and after treatment.

Section-6: In this section, the *in vitro* production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-17, IFN-γ and TNF-α and regulatory cytokine (IL-10) in PBMCs in response to the stimulation with *M.leprae* whole cell sonicate in the patients with ENL and LL controls before and after treatment are presented.

Section-7: The gene expression level of the above cytokines in the blood as well as in the skin biopsies of ENL and LL is described in section-7.

Section-8: Gives preliminary data on the circulating C1q and their genes expression level in blood and skin biopsies of patients with ENL and LL controls before and after treatment.

Section 9: This section presents the levels of anti ND-O-BSA (PGL-1), Ag85 protein and LAM antibodies in the plasma samples of patients with ENL and LL controls before and after treatment.

In chapter-7 the main findings of the study are discussed. Chapter-8 presents conclusions, recommendations and future directions for the study of ENL. Finally, my roles in this thesis include: writing the grant for the project under the guidance of my supervisor, developing laboratory standard operating procedures, obtaining ethical clearance, identifying consumable laboratory reagents and antibodies, processing procurements of laboratory consumables, obtaining permits and support letters from different institutions for shipping the reagents to Ethiopia, designing framework for study subjects recruitment and follow-up, documentation of clinical data; laboratory assays (PBMCs isolation, freezing, storage and thawing; RNA extraction; ELISA; flow cytometry and qPCR); data entry, analysis and interpretation and write-up of the thesis, controlling financial expenditures at the study site (payments for biopsy nurses, runner, compensation for study subjects) and others.

CHAPTER 2: LITERATURE REVIEW OF LEPROSY

2.1. Literature search strategy and search criteria

To review the Immunopathology of Erythema Nodosum Leprosum (ENL), literature published up to May 2016 was searched. The literature review of ENL and leprosy was performed using keywords or phrases "Leprosy", "Hansen's disease", "lepr", "leprosy reactions", "type 1 reaction", "reversal reaction"," Type II reaction", "Erythema nodosum leprosum","ENL", "Lepromatous leprosy, "LL" ,"Leprosy spectrum" , "MDT", "prednisolone", "leprosy immunology", "immunopathology", "immune-complex", " regulatory T cells" "B cells", "Memory T cells", "Memory B cells", "cytokines", "leprosy treatment", "*Mycobacterium leprae"* "*M. leprae*". The search was limited to articles published in English. Search engines and bibliographic data-base were the main research resources used for this thesis. PubMed and Cochrane were used to obtain articles with wide coverage of health topics and clinical trials related to leprosy respectively. Medline was used to obtain subject guided articles in the area of medical and biomedical sciences. EMBASE were used to pick recent articles, conference papers, and articles which included drugs and pharmacy topics. World Health Organization (WHO) documents on leprosy were checked on the WHO websites. Additional references from professional associations, conferences and google internet search were also included. ETHOS database was used to search PhD doctoral theses available in UK. LSHTM and UCL libraries were also checked for relevant information. Among the 3,789 articles identified for the immunology of leprosy, 477 of them were considered for the current review (Table 2.1).

Table 2.1. Summary of the number of articles retrieved on leprosy research from database search (1920- 2016).

Category	No of publications
All leprosy research articles since 1920	27,162
Leprosy immunology	8,789
Leprosy reactions	4,912
Immunology of leprosy reactions	2,462
Erythema Nodosum Leprosum (ENL)	1,986
Immunology of ENL	890
All leprosy related studies in Africa	2,168
All leprosy related studies in Ethiopia	281

2.2. Historical aspects of leprosy

2.2.1. Global aspect

Leprosy is generally considered as one of the oldest diseases known to man. Understanding the origins of leprosy is important both epidemiologically and historically to describe the human interactions as well as the biological and social reaction to the disease. However, the origin of leprosy is not precisely known. It has been found that around 600BC an Indian writings describe a disease that resembles leprosy as reviewed by Pankhurst (1984). The most ancient evidence of leprosy comes from a 4,000-year-old human skeleton uncovered in India in 2009 which proved that leprosy existed in India before 2000BC (Robbins et al., 2009). Symptoms that are used today to describe leprosy were documented in Chinese books in 400 BC (Pankhurst, 1984). Greek writers described a disease that may have been leprosy in the 2nd century BC(Susannah, 2015) .

According to the historians, in Europe leprosy first appeared in the records of ancient Greece after the army of Alexander the Great came back from India in 4th century BC (Browne, 1975) and carried it into the Middle East and then throughout the eastern Mediterranean upon their return home from Egypt (Lechat, 1999). It is also traditionally believed that Roman soldiers in the army of Pompey took the

disease from Egypt to Italy in 62 BC and that Roman armies later took the disease as far as the British Isles in 60 BC (Browne, 1975, Susannah, 2015).

Archaeological studies of Egyptian skeleton have shown that leprosy was prevalent in Egypt during the 2nd century BC and claimed that leprosy conceivably originated in Egypt. Other scholars, suggested that the disease probably originated in India and spread to Europe after the 4th century B.C. (Lechat, 1999). Genetic analysis of the leprosy bacillus has shown that *M. leprae* very likely evolved some 100,000 years ago in eastern Africa or South-western Asia and then would have speared to other areas through human migration and colonization to Europe and North Africa. The introduction of leprosy to America is assumed to be the result of colonization by Europe or by the slave trade from West Africa in the 18th century (Monot et al., 2005b).

2.2.2. **Leprosy in Ethiopia**

Historically the existence of leprosy in Ethiopia had been documented by traveller literature after the medieval period. It is not known when and where the disease was first diagnosed in the country. Pankhurst has mentioned that Ethiopians has suffered since time of immemorial from a high burden of leprosy in his article "*the history of leprosy in Ethiopia to 1935*" (Pankhurst, 1984). However, the author did not mention the time and the magnitude of the disease.

In the 16th century, a Portuguese priest, Fransisco Alvares was the first person to document the high incidence of 'lepers' in Ethiopia. A British traveller, Nathaniel Pearce, had reported in 1831 that leprosy was common in the northern country among lower class and thousands of them had lost their fingers and toes. Many travellers from France, Germany and Italy had also reported a high prevalence of leprosy in north, south and central Ethiopia in the 1840s (Pankhurst, 1984).

In the past, it was believed that leprosy was hereditary disease or an act of God among Christians in Ethiopia. It was also commonly believed that leprosy was caused by the devil, through adultery and or by being possessed by spirits. Affected persons would pray at churches to be cured of this disease, wear amulets around their necks, take medicated herbal baths and immerse themselves in thermal pools.

They also tried using herbal medicines, both internally and externally (Pankhurst, 1984).

Leprosy had been known in Ethiopia, since early times, as *lamts*. *Lamts* is a word found in the Old Testament of the bible which was translated into Ge'ez, one of the Semitic languages in Ethiopia. However, leprosy is also often designated by general terms which are also applicable to elephantiasis or other serious skin diseases (Pankhurst, 1984). Leprosy is formally known as *Sega Dawe* in Amharic which means ulcerated or diseased *sega* (body). Frequently leprosy is referred to more explicitly as *Qumtena*, an Amharic word derived from the verb, to amputate. A similar concept is used among the Oromos, the most populous ethnic group in Ethiopia. The Oromo people refer leprosy as *Kurchi*, a word whose origin in the verb, to cut or break (Pankhurst, 1984).

It has been assumed that the legends and beliefs (Christianity) formed the attitude of the Ethiopian society towards leprosy. They are considerably more tolerant and compassionate towards people affected by leprosy than that of the West, where "total exclusion" of people affected by leprosy in the early medieval period had been followed by their rigid detention in "houses of Lazarus" as reviewed by Pankhurst (1984).

The Ethiopians attitude toward leprosy has been probably shaped by the country's traditional code, the *Fetha Nagast* (Law of the Kings) which took a fundamentally empirical and humanitarian view of this disabling disease. However, it was declared that a person affected by leprosy could not serve as a Priest or Patriarch not because of uncleanness but cause priest to be unloved. Similarly, it was declared that a judge had to be free of leprosy only because the infection keeps people away who may want to see the judge (Pankhurst, 1984).

Nevertheless, leprosy patients faced stigma which resulted in physical isolation and discrimination in some areas even recently (Tekle-Haimanot et al., 1992). In some places they are forbidden to beg during the day and are forced to move at night from house- to- house to request for alms. They move in group to protect themselves from wild animals and dogs. The group is called *Hamina* and the H*amina* group practice the *Hamina* song-mendicant believing that it will ward off from debilitating

by the disease and to get relieve from the symptoms of the disease (Kebede, 2010). To date the *Hamina* descendants firmly believe that they own an inherited right of song-mendicancy which they think as their social and traditional duty. They believe that they will be stricken with leprosy if they did not sing. Moving from house- to-house and from door-to-door singing the *Hamina* song, suffering from the chilly nights and being attacked by barking dogs is a mandatory ritual ceremony for them at least once a year (Kebede, 2010).

At the beginning of 20th century, information that leprosy was a contagious disease arriving in Ethiopia through missionaries and thus, the idea of isolating leprosy patients from the community started to be introduced to some extent as a means of encumbering the spread of the disease. With this idea the first Ethiopian leprosarium was established at Harar named, *Ras Makonnen's Leprosarium***,** in 1901. The establishment of *Ras Makonnen's Leprosarium* was funded by French catholic missionaries and at the beginning it was run entirely by French missionaries giving full service for 25 leprosy families. *Ras Makonnen's Leprosarium* is now called *Bisidimo* leprosarium. The second was opened in 1934 at *Akaki*, just outside Addis Ababa. In the 1940s and 1950s, a total of 11 leprosaria were opened within a few years: *Yesilase leprosurium* was opened in *Borumeda* of *Woll*o province, *Tibela* in *Arusi*, *Kuyera* and *Gambo* in *Shashemene, Finoteselam* in *Gojam* province, *Gindeberet* in *Shewa province*, *Hosaina* in Shewa province, *Akaki* and *Zenabewark* in Addis Ababa. In the 1950s leprosy was identified as a major public health problem in Ethiopia. Leprosy control program was started in limited areas of Ethiopia in 1956 (Berhe et al., 1990).

A leprosy community grew around *Zenebawark* (ALERT) Hospital made up of people who had come from all regions of Ethiopia. Many patients stayed around ALERT Hospital after they finished their leprosy treatment because they felt unable to return home fearing the stigma and social exclusion. This community quickly grew with new arrivals and new generations born it to the area and the village has got its name *Kore.* In an attempt to limit the size of the *Kore* community, the government forcibly moved many people with leprosy to remote, isolated areas (*Addis Hiwot* and *Tesfa Hiwot*) and to Kuyara in 1954 (Mesele Terecha, 2005).

The people remaining in the *Zenebawark* area relied heavily on ALERT and the facilities it provided, which contributed to the dependency attitude formed with local leprosy affected people enjoying access to free, on demand healthcare. However, ALERT began change its method of operation advised by the donors to restructure its funding arrangements because the system in which it was operating was costly and unsustainable but the *Kore* community did not understand the situation. This situation accounted for the establishment of Addis Ababa Association of Ex-Leprosy patients now named Ethiopian National Ex-leprosy Association (ENAELP) in 1992 (Isabelle, 2005).

Currently, leprosy treatment is integrated to the general health system in the country. Suspected patients are seen at health posts and then referred to the health centres. Most clinically confirmed patients with leprosy are treated with MDT at health centres or referred to district and zonal hospital for further diagnosis. The leprosy referral systems in Ethiopia will be explained in section 2.4.

2.3. Epidemiology of Leprosy

2.3.1. Definitions

Leprosy is a chronic granulomatous infectious disease mainly affecting the skin and peripheral nerves. The etiologic agent of leprosy is *Mycobacterium leprae*, an obligate intracellular bacterium as reviewed by Lockwood (2004). A case of leprosy is defined as a person with one or more of the following features, and who has yet to complete a full course of treatment: i). hypopigmented or reddish skin lesion(s) with definite loss of sensation ii) damage to the peripheral nerves, as demonstrated by loss of sensation and mobility to hand, feet or face iii) positive slit-skin smears for acid fast bacilli (World Health organization, 1998). This case definition does not include cured persons with late leprosy reactions or with residual disabilities.

2.3.2. Global Prevalence of leprosy

The global prevalence of leprosy has decreased from more than 5 million cases in the mid-1980s to less than 200,000 cases in 2015. A total of 175,554 registered prevalence of leprosy cases were reported to WHO in the beginning of 2015 with 213,899 new case detections from 121 countries (WHO, 2015). According to the

WHO report, the introduction of multi-drug therapy (MDT) for the treatment of leprosy, improved early diagnoses and complete treatment with MDT are among the key factors which contributed to the reduction of disease burden.

The registered prevalence rate in the beginning of 2015 was 0.31 per 10,000 populations which is less than that of 2014 (0.32 per 10,000 populations). However, the number of new cases reported at the end of 2014 (3.78 per 100,000 populations) was almost at the same level as in the previous year (3.81 per 100,000 populations). Furthermore, a new case detection prediction model by Smith et al. (2015) has reported that over 2.6 million cases are missed during 2000 and 2012 which implies that there may be a large accumulation of people with leprosy in the community who remain undiagnosed. At the end of 2014, thirteen countries: India, Brazil, Indonesia, Ethiopia, Bangladesh, Democratic Republic Congo, Nepal, Myanmar, Nigeria, Sir Lanka, Tanzania, Madagascar and the Philippines reported more than 1,000 new cases in 2014. These 13 countries together contributed 94% of all new leprosy cases worldwide (WHO, 2015).

From the total new leprosy cases reported, 72% were from South-east Asia, 16% from South America and 9% were from Africa. The highest new leprosy cases detection was reported from 8 countries. These are: India (125,785 new cases), Brazil (31,064), Indonesia (17,025), Ethiopia (3,758), Bangladesh (3,622), Democratic Republic Congo (3,272), Nepal (3,046) and Nigeria (2,983). India, Brazil and Indonesia reported greater than 10,000 new cases in 2014 and they accounted for 81% of new leprosy cases globally. Ethiopia, Democratic Republic of Congo, Madagascar, Nigeria, Tanzania, Bangladesh, Myanmar, Nepal, Sir Lanka and Philippines reported between 1000 and 10,000 new cases in 2014 and they accounted for 13% of new leprosy cases in the reported year. The remaining 108 countries all together contributed only 6% of all new leprosy cases (WHO, 2015).

It is very difficult to estimate the actual prevalence of leprosy due to the long incubation period of *M. leprae,* delays in diagnosis after onset of the disease, and the lack of laboratory tools to detect leprosy in its very early stages (WHO, 1985). Instead, the registered prevalence is used. Registered prevalence in leprosy is defined as the number of patients registered for MDT treatment during the reported year per 10,000 populations. Incidence rate or new case detection rate in leprosy is

defined as the number of newly diagnosed leprosy cases per 100,000 populations per year. Incidence is the most useful transmission index to monitor the success of leprosy control program since it reflects the current risk of developing leprosy within a specified population. New leprosy case detection among children provides better information that there is an early and continued transmission of infection in the community. The proportion of children under 15 years among newly diagnosed leprosy cases globally in 2014 was 8.8% with a range of 34.5 % in Comoros to 1.4% in Niger (WHO, 2015).

According to the WHO report, although the prevalence of leprosy has been declining (Figure 2.1) due to the introduction of MDT and reducing the treatment time, new cases continue to occur in almost all endemic countries. The "enhanced global strategy for further reducing the disease burden due to leprosy from 2011 to 2015" is being implemented by national programmes in endemic countries. One of the aims of the strategy is to reduce the global rate of new cases with grade-2 disabilities (i.e. visible deformity and damage) per 100,000 population by at least 35% by the end of 2015, compared with the baseline at the end of 2010. However, the trend of new cases with grade-2 disabilities (G2D) and rates per 100,000 populations did not change significantly from 2005 to 2014 (Figure 2.2). In 2005 the grade-2 disabilities among the new cases was 0.25 per 100,000 population and it was the same in 2014 (0.25). The proportion of new G2D ranges from 28.0% in Uganda to 0.0% in Kenya with global average of 6.6% in 2014 (WHO, 2015).

The level of new G2D cases is the indicator of delayed diagnosis and treatment of leprosy. The rate of new cases detected with grade-2 disabilities per 100,000 population is proposed as the indicator for a global target by WHO since it is less influenced by operational factors; focuses attention on impairments which are critical to persons affected by leprosy, and stimulates improvements in case detection. Delayed diagnosis and treatment of leprosy could be happened either due to lack of awareness about the early signs of leprosy and the health seeking response in the society or lack of the capacity in the health system to diagnose and treat leprosy in time before the development of disabilities.

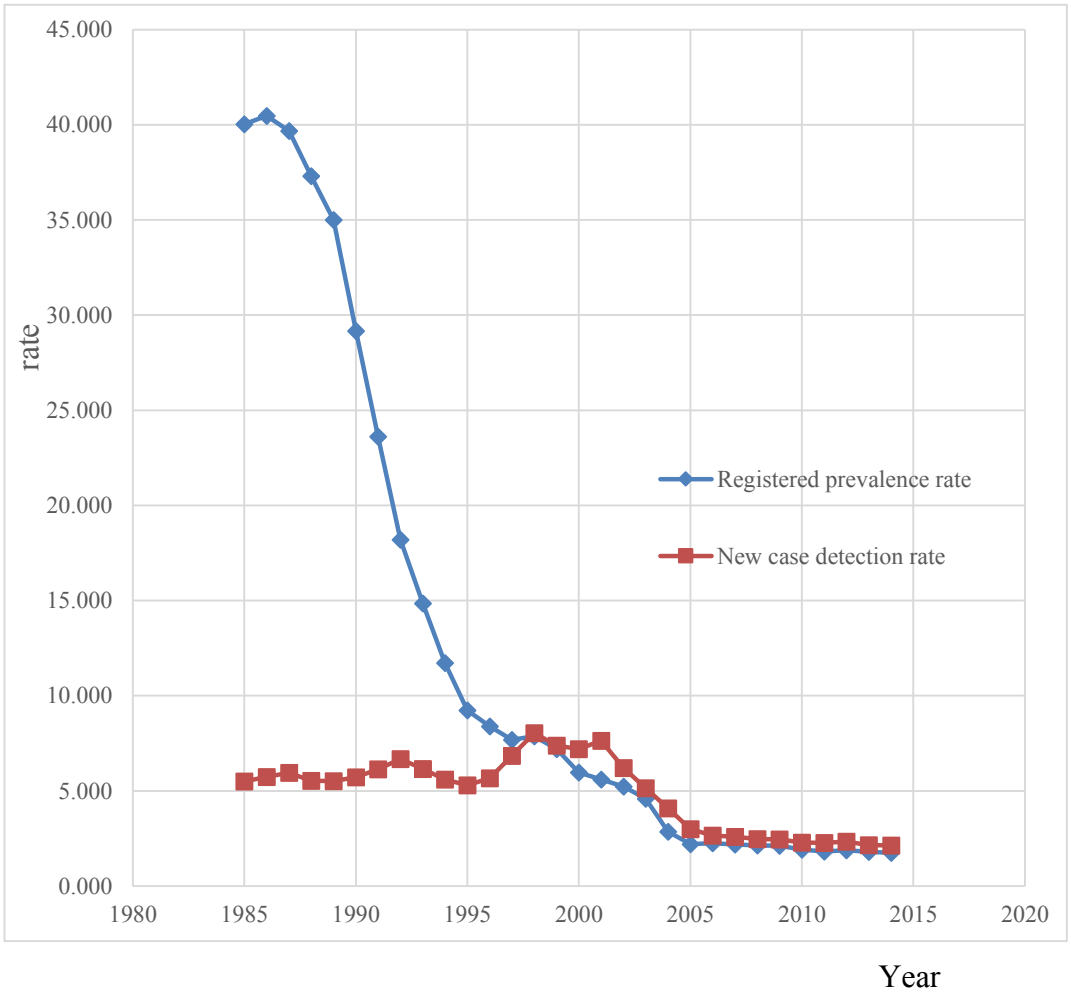


Figure 2. 1. Global Registered prevalence and new cases detection rates of leprosy per 100,000 population from 1985 to the beginning of 2014. Figures generated from WHO data series

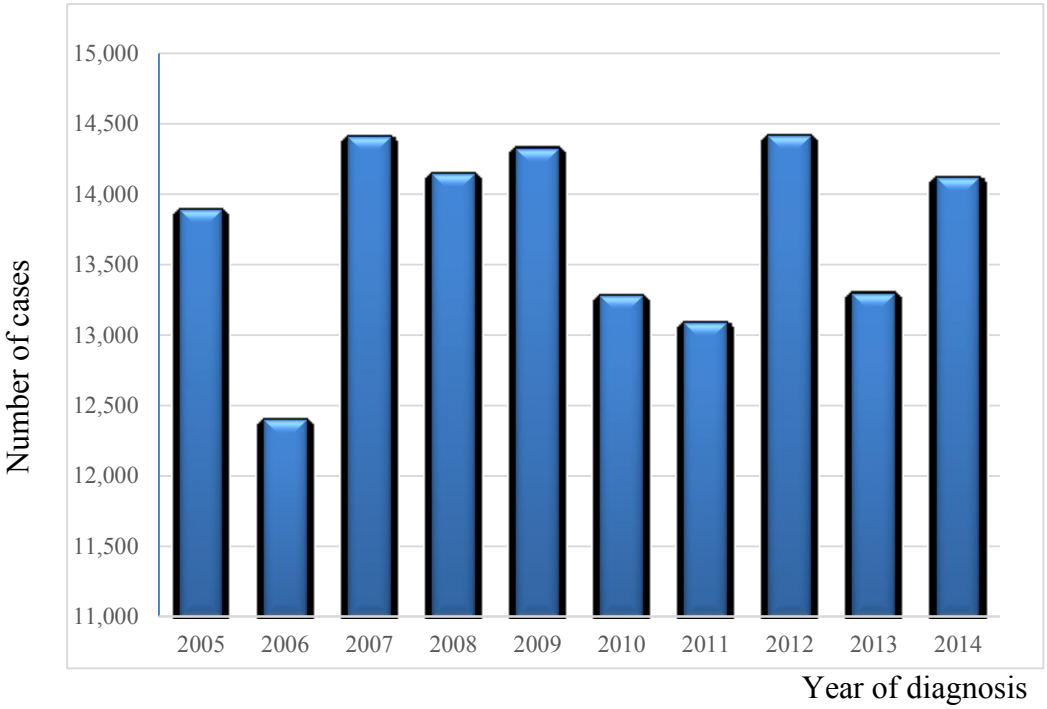


Figure 2.2 Number of new grade-2 disabilities among newly detected leprosy cases reported to WHO from 2005-2014.
Figures generated from WHO) data series: http://www.who.int/gho/database/en/

2.3.3. Epidemiology of leprosy in Ethiopia

Registered prevalence of leprosy cases in Ethiopia: The prevalence and the incidence of leprosy in Ethiopia are not precisely known because not all regions report registered cases to Ministry of Health regularly. According to the reports of the Ministry of Health, leprosy notification rate in Ethiopia remained stable over the last five years at between 4000 and 5,000 new leprosy cases recorded per year (Figure 2.3).

In 1982, before the introduction of MDT to Ethiopia, 80,927 registered leprosy cases were reported to WHO. Following the introduction of MDT in 1983, the registered prevalence dropped to 59,822 cases in 1984. The registered prevalence declined rapidly and reached 12,041 cases at the end of 1990 and increased to 16,670 cases in 1991. In 1992 the registered prevalence slightly dropped to 15,673 cases and reached 12,698 in 1993. However, after 1994 the registered prevalence had decreased slightly and dropped to 3,758 registered cases after 2 decades in

2014. This means since 1994, the registered prevalence on average had declined by 293 cases every year (Figure 2.3)

New case detection of leprosy in Ethiopia: unlike the registered prevalence, figure 2.3 shows a slow decline in new cases detection rate since 1982. In 1982, 6,243 new leprosy cases were diagnosed. This number dropped to 3,758 cases after 3 decades (Figure 2.3). On average 4,018 new leprosy cases (0.04/100,000 population) were detected for the last 33 years. However, it should be noticed that there could be more new leprosy cases in Ethiopia than annually reported according to "The Missing Millions" predication model of Smith et al (2015).

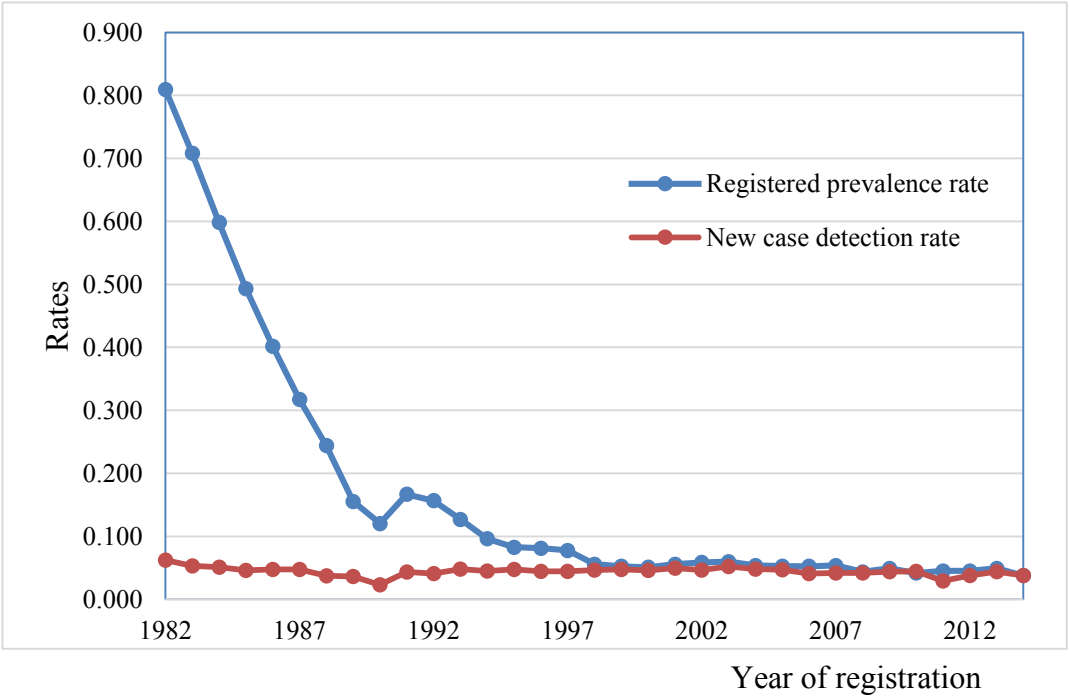


Figure 2.3. Registered prevalence and new cases detection rates of leprosy per 100,000 population in Ethiopia from 1982-2014. Figures generated from WHO and Ethiopian Federal Ministry of Health (EFMOH) data series

Multibacillary (MB) and Paucibacillary (PB) cases: Most leprosy cases reported in Ethiopia are MB cases. For the past 15 years (2000-2014), on average 4,410 (89.5%) registered MB cases has been reported. The overall ratio of registered MB to PB cases indicated that MB cases were nearly nine-folds of PB cases during these periods. The proportion of MB cases increased from 2000 (82.1%) to 2014 (91.8%) among registered cases (EFMOH, 2015).

On average 4,308 new leprosy cases were diagnosed per year from 2000-2014 of which MB cases accounted for 86%. Similar to the registered leprosy cases, the proportion of MB cases in newly diagnosed leprosy cases showed an increasing trend over these periods except in 2014. In 2014 the proportion of MB cases strangely dropped to 73.7% among newly diagnosed leprosy cases in the year (EFMOH, 2015).

2.3.4. Treatment outcome, defaulter rate and relapse cases in Ethiopia

On average 4,815 leprosy patients annually had been treated with MDT from 2000-2014 (EFMOH, 2015) and the MDT completion rate over these years was 84.4% The treatment completion rate increased from 78% in 2000 to 89% in 2014. The average annual defaulter rate from 2000-2011 was 3.7%. Data was not available for the years 2012-2014. The defaulter rate had significantly dropped from 4% in 2000 to 1.5% in 2011. However, the reduction did not occur in a steady state rather in two stages (decreasing and increasing trend) over these periods. The increasing and then decreasing trends of defaulter rate could be due to several factors. It could be due to underreporting, shortage of MDT during that particular year or some districts might not reported at all to the regional health bureau. On average, 212 relapse cases per year with an overall rate of 3.8% were reported. Relapse, in MB leprosy, is defined as the multiplication of *M. leprae*, suspected by the marked increase (at least 2+ over the previous value) in the BI at any single site, usually with evidence of clinical deterioration (new skin patches or nodules and/or new nerve damage). The relapse rate had increased from 2.4% in 2000 to 7.4% in 2010 (EFMOH, 2015) which raises questions about the future effectiveness of MDT. However, relapse case is difficult to determine because it should relate to the year when MDT was given.

2.3.5 Childhood leprosy and grade-2 disability in Ethiopia

Childhood leprosy: The average proportion of childhood leprosy (0-14 years) in Ethiopia is 6.3% for the registered leprosy cases and 7.1% for the newly detected cases (Baye, 2015). The rate of childhood leprosy among newly diagnosed leprosy cases had increased from 8% in 2006 to 12.8% in 2014 (Baye, 2015). The increasing rate of childhood leprosy indicates high level of transmission in Ethiopia. Lack of technical means of interruption transmission may further intensify the possibility of

leprosy transmission in the country. However, the increasing rate of childhood leprosy in the country could be also due to the higher coverage of health service after the integration of leprosy to the general health service program.

Total grade- 2 disability rates: In Ethiopia leprosy patients present with either grade-1 or grade-2 disabilities at the diagnosis of leprosy (Baye, 2015). On average about 9.8% of leprosy cases had grade-2 disability (G2D) at the time of diagnosis of leprosy from 2005 to 2014 (Figure 2.4). In 2005 about 15% of leprosy cases were diagnosed with G2D which declined to about 10% in 2014. The fall in G2D rate, however, seemed to have occurred gradually in different stages; a slight fall in the beginning followed by sharp rise in middle and a relatively gradual rise in the end (Figure 2.4) which signals the need for improving early case detection system in the country.

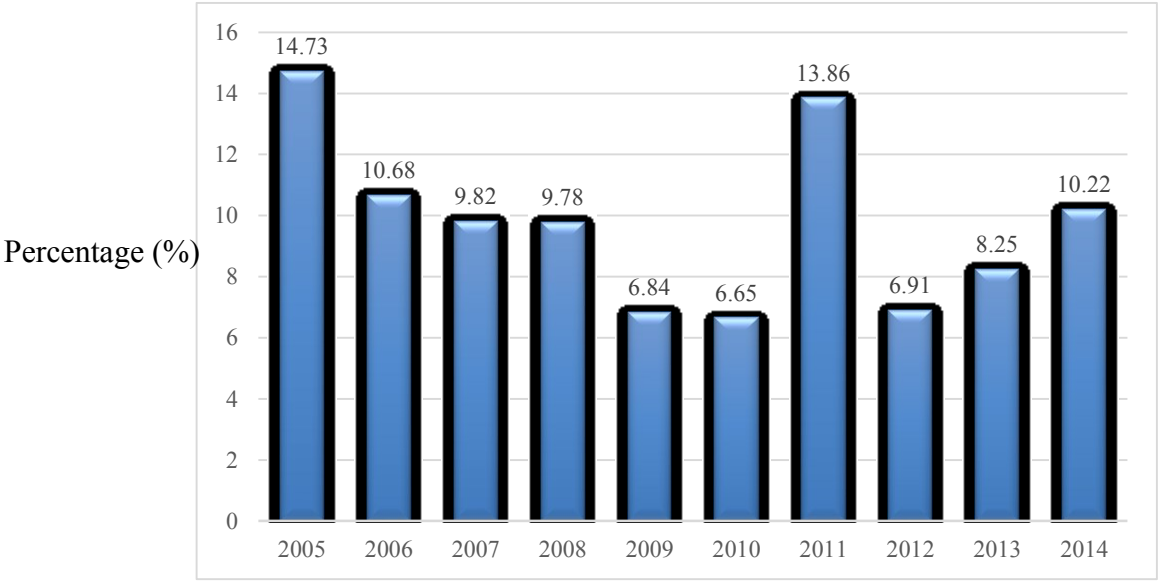


Figure 2.4. Trends of grade-2 disability (G2D) among newly diagnosed leprosy cases in Ethiopia from 2006 - 2014. Data compiled from EFMOH (2015)

2.4. The Ethiopian Health system

Ethiopia has an estimated population of 84.32 million with median age of 18.6 years and life expectancy of 64 years (CSE and ICF, 2012). Ethiopia has a federal system where power is decentralized to nine regional states and the city administration councils of two cities. The administration hierarchy in each regional states follows the regional levels, zonal and woreda (district) and finally the Kebels. Kebele is the smallest administrative level.

The health system in Ethiopia is a three-tier health system with special attention to the health extension program which runs under the primary health care delivery (Figure 2.5). The health extension program serves as the primary vehicle for implementation of community-centred essential health care packages and as an effective referral system from the grass-roots level to broaden access to care at secondary and tertiary levels. The first level is the district health system consisting of one primary hospital, 5 health centres and 25 health posts. The second level is the general hospital serving for one million people and the third level is a special led hospital giving service for 5 million people. Ethiopia has 149 hospitals, 2,953 Health centres and about 16,000 health posts (EFMOH et al., 2015).

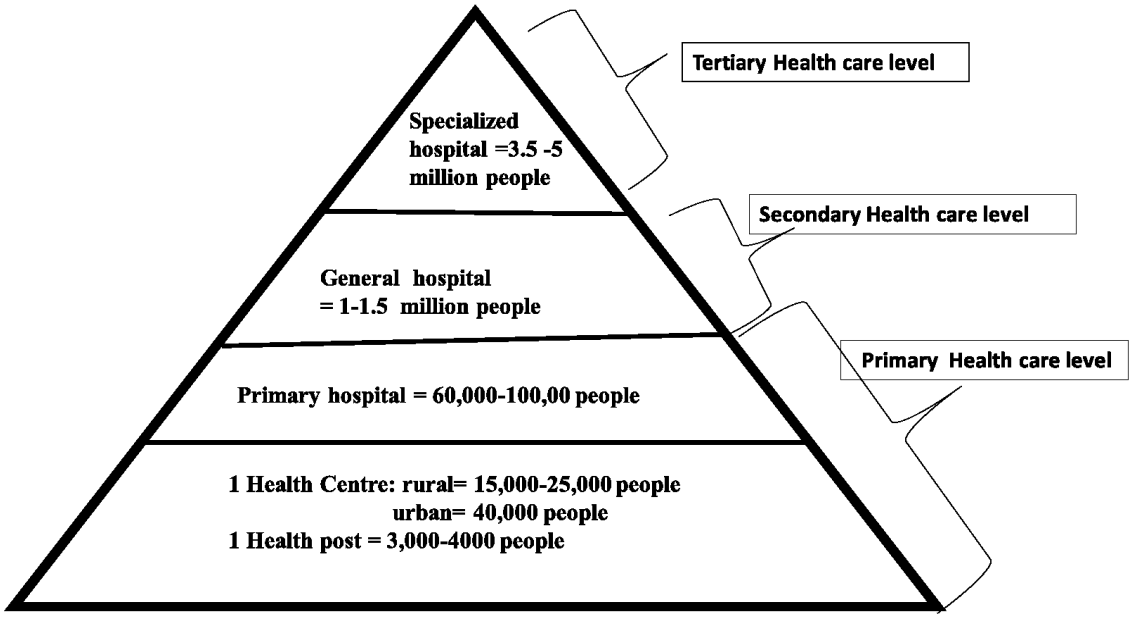


Figure 2.5. Ethiopian three-tier health system. Data source: EFMOH (2015).

2.4.1. Leprosy treatment situation in Ethiopian health system

In Ethiopia health care system, leprosy was treated vertically by leprosy specialized personnel until the control program was fully integrated into the general health services by the end of 2001. The need for integration was to ensure that patients are diagnosed at an early stage and complete the multidrug treatment (MDT) before disability ensues. This means that leprosy prevention and control activities became the responsibility of the general health service. However, this integration meant that patients are seen by general health workers during outpatient visits rather than by leprosy specialized personnel in leprosy dedicated clinics. Hence, the diagnosis of leprosy might be delayed because of poor recognition of early signs or lack of a high index of suspicion (Abeje et al., 2016). In reality, the health staff training on leprosy is inadequate and can be easily outstripped by the high prevalence of TB and HIV in the country because more attention is given to these diseases.

From health posts leprosy suspects are referred to health centres. Health centres diagnose and treat leprosy patients and are the main health facility for initiating MDT and follow-up. They are also supposed to treat and manage reactions and refer severe reactions and complications to hospitals. In fact, steroids for treatment of reactions are only available at hospitals. Leprosy referral hospitals provide referral services for diagnosis and treatment and provide in-patient services. These leprosy referral hospitals are quite few and deal only with complicated cases such as leprosy reactions. Drugs are supplied quarterly to health facilities based on the previous quarter registered number of patients. Regular assessment is done at the Woreda, zonal, regional and national levels where epidemiological and operational indicators for monitoring of the leprosy are calculated and compiled. Quarterly reports are completed according to the Ethiopian fiscal year. Tuberculosis (TB), Leprosy and TB/HIV collaborative activities eventually integrated into the Health Management Information System.

2.4.2. Leprosy treatment and management at ALERT Centre

ALERT is a medical facility located in Addis Ababa. ALERT was originally the All Africa Leprosy Rehabilitation and Training Centre (hence the acronym), but the official name is now expanded to include tuberculosis: All Africa Leprosy and,

Tuberculosis Rehabilitation and Training Centre. It is the main leprosy specialized hospital in Ethiopia. The activities of the hospital included diagnosis, treatment and rehabilitation of leprosy patients, provides training programs for leprosy personnel from around the world, and leprosy control (administration of the Ethiopian Ministry of Health's regional leprosy control program). ALERT is the continuation and expansion of the leprosy hospital originally built by Dr Thomas Lambie in 1922, which was later named the Princess *ZenebeWor*k Hospital, the second daughter of Emperor Haile Selassie of Ethiopia.

The Armauer Hansen Research Institute (AHRI) is a biomedical research institute which was established by the Swedish and Norwegian Save the Children organisations in collaboration with the University of Bergen, with the principal objective of pursuing basic research in leprosy. AHRI is located in the same compound with ALERT. While AHRI was engaged in leprosy research, the ALERT hospital was fully involved in leprosy treatment and management until the portfolio of the two institutions expanded to include other infectious diseases. Both ALERT and AHRI are managed by the Ethiopian Federal Ministry of Health.

Leprosy patients are seen at ALERT hospital at Red Medical Clinic. The clinic is staffed with a few dermatologists and nurses.

Figure 2.6. Pictures of ALERT and AHRI. (A) ALERT in 1960s, (B) Present picture of ALERT, (C) AHRI, (D) A nurse taking history from leprosy patient at Red Medical Clinic (RMC). For the patient picture, a consent has been obtained

2.5. Transmission

Anthroponotic Transmission: Transmission of leprosy from an infected person to a healthy person is the most accepted mode of transmission by scientists. Untreated multibacillary cases, especially lepromatous patients are the most important source of infection compared to the other types in the spectrum. This is because lepromatous patients harbour significantly higher numbers of bacilli. Various investigators have also reported that household contacts are at a greater risk of developing leprosy compared to the general population.

In Northern Malawi, 80,000 initially leprosy free individuals in leprosy endemic area were followed for 10 years and 331 of them developed leprosy (Fine et al., 1997). In this study, it was found that the risk of household contacts of MB patients was eight-fold and that of PB patients two-fold compared with individuals not living with leprosy indexes. The risk was greatest for those household contacts living together than visiting household contacts of MB patients. Such association was not found in PB patients suggesting that PB cases may not be the source of infection, but rather an indication that households have had contacts with a source of infection (Fine et al., 1997).

In a retrospective study conducted in Indonesia, the relative risk of household and neighbourhood contacts was found to be 8 and 4 times, respectively, compared to households that had had no such contact with leprosy patients (van Beers et al., 1999). Similarly, the relative risk of leprosy among household contacts was found to be 8 times more than the general population (Vijayakumaran et al., 1998). Close household contacts such as parents, siblings and children have a higher risk of contracting leprosy than other type of household contacts (Vijayakumaran et al., 1998, Deps et al., 2008). A study conducted in the Philippines which included 2,087 household contacts and 4,750 community contacts, the relative risk of household contacts was 26 times that of the community contacts (Cunanan et al., 1998).

Although the portal of entry and mode of spread of *M. leprae* in human leprosy is not scientifically proven, the nose is considered to be the main portal of exit and

entry of *M. leprae* and hence suggesting an aerosol route of transmission through droplet infection. An assessment of the presence of *M. leprae* on the nasal mucosa in general population in leprosy endemic area was conducted in India. They found that among 400 healthy inhabitants, about 31% of them showed PCR positivity for *M .leprae* DNA highlighting the aerosol route of transmission of leprosy through droplet infection (Lavania et al., 2013). Another larger study which included 444 leprosy patients and 1,352 healthy household contacts in Brazil, has reported that the nasal swab of 4.7% household contacts were positive for *M. leprae* DNA (Araujo et al., 2012). An experimental infection of mice has shown that among the 30 mice exposed to *M. leprae* aerosols 33% of then had countable numbers of acid-fast bacilli (greater than 2 $x10^4$ with the characteristics of *M. leprae* in one or more homogenates prepared from ears, foot pads, nose or lungs (Rees and McDougall, 1977). These authors has shown that from the distribution of *M. leprae* that the infection had arisen from systemic spread of bacilli initially entering the lungs rather than from multiplication of organisms locally retained there, or in the nose, at the time of airborne infection.

However, various investigators suggested that skin could also be another source of infection. A clinical and bacteriological examination of *M. leprae* in the epidermis and cutaneous appendages of patients with multibacillary leprosy revealed that 11% of samples were positive for acid-fast bacilli (AFB) (Hosokawa, 1999). The author, found that the AFB positivity rate was higher in untreated non-ulcerating skin lesions with minor inflammation or in lesions with leprosy reactions than typical skin lesions such as papules, nodules and infiltrated punched out skin lesions indicating that these lesions could be one possible source of infection.

Similarly, Job et al. (2008) reported that both skin and nasal mucosa could contribute to the shading of *M. leprae* into the environment and hence transmission. These authors evaluated *M. leprae* in the unbroken skin and nasal secretions of multibacillary leprosy patients and their contacts at St Thomas Hospital and Leprosy Centre Chettupattu, India. About 60% of untreated MB leprosy patients examined histopthologically had *M. leprae* in the keratin layer of the skin. Using PCR, *M. leprae* DNA was detected in the skin washings and nasal swabs in 80% and 60% of these patients respectively. They also confirmed the presence of *M.*

leprae DNA in healthy contacts (17% in the skin and 4% in the nasal swabs) before initiating treatment of index cases and after treating the index cases for 2 months the contacts tested negative for *M. leprae* DNA (Job et al., 2008). Similarly, the presence of *M. leprae* in intact skin of patients with leprosy has been reported by Satapathy et al. (2005) and Satapathy et al. (2005). A case-control study in a hyper-endemic region of north-eastern Brazil, found that 69.2% (116/185) of leprosy cases and 66.9% (91/136) endemic controls without leprosy were positive for *M. leprae* DNA in the nasal secretions (Lima et al., 2015).

In view of the above findings, it seems appropriate to consider that the skin is at least one of the important routes of transmission of the disease and that infection can occur through skin to skin contact with leprosy patients, especially with those in the lepromatous leprosy spectrum, in addition to the aerosol spread of nasal secretions and uptake through nasal or respiratory mucosa.

Zoonotic transmission: It was believed that man is the only natural reservoir host of *M. leprae* and the only source of infection is an untreated case of leprosy. However, recent investigations have indicated that a nine banded armadillo (*Dasypus novemcinctus* L) is also natural reservoir of *M. leprae,* reviewed by Turman (2005).

A temporal aspects of leprosy infection in a wild population of nine-banded armadillos in Western Mississippi from 2005-2010 reported an annual prevalence of infection varied between 4.5–15% (Williams and Loughry, 2012). In this study the incidence density estimates calculated over progressively longer time intervals generated values ranging from 0.11–0.61 new cases of infection/1000 days indicating that a substantial potential for transmission of infection within the armadillo population.

In the southern region of the Unites States of America, such as in Louisiana and Texas, cases of leprosy among native-born Americans with no history of foreign exposure has been commonly reported (Truman et al., 2011). In the same region, a wild armadillo infected with *M. leprae* has been confirmed. A comparative genomic analysis of *M. leprae* obtained from 50 patients and 33 wild armadillos from southern USA, confirmed that both humans and armadillos were infected with the

same strains of *M. leprae* (Truman et al., 2011). These authors isolated nearly identical strains of *M. leprae* from 28 of 33 wild armadillos and from 25 of the 39 patients who lived in areas of the USA where they could be exposed to armadillos. They found different strains in people who were infected in the Philippines and Brazil. This finding strengthens the long-held hypothesis that armadillos can be a source of infection in humans and that leprosy is probably a zoonotic disease in the southern United States. It is important to mention that the risk of getting leprosy from armadillo is described as "low" by these investigators.

A case-control study conducted in Brazil suggested that direct exposure to armadillos is a risk factor for leprosy in Brazil (Deps et al., 2008). Deps et al., included in their survey, a total of 1,100 peoples (506 cases with active leprosy and 594 health controls). They assessed the history of direct exposure to armadillos through structured questionnaires. The result of the study has shown that direct exposure to armadillos is associated with a two-fold increase in the incidence of leprosy in the study area.

2.6. Microbiology of *M. leprae*

Mycobacterium leprae is an acid fast, non-motile, *Mycobacterium* which belongs to the class Actinomycetales and the genus *Mycobacterium* based on the cell structure, staining properties and chemical composition of the cell wall, presence of mycolic acids, antigenic characteristics of mycobacteria and a lipid-rich cell envelope (Monot et al., 2005a, Shinnick, 2006). Morphologically the bacterium is straight or slightly curved bacillus (road shape) having a length of 4 to 8µm and 0.3 to 0.5µm diameter. *M. leprae* exists as single or clump of masses. It does not have capsules, flagella and spores (Shinnick, 2006). The cell of *M. leprae* is bounded by unique cell wall which defines the cell shape and provides protection from environmental stress. The cell wall of *M. leprae* is structurally similar to that of most *Mycobacteriu*m species. The cell wall is composed of two layers, the outer and inner layers. The outer layer contains lipopolysaccharide made up of branching chains of arabinogalactan esterified with long chain of mycolic acids. The inner layer is made up of peptidoglycan (Figure 2.7). The cell membrane of these bacteria is composed of lipids and protein like other bacteria. Within the cytoplasm there are few organelles.

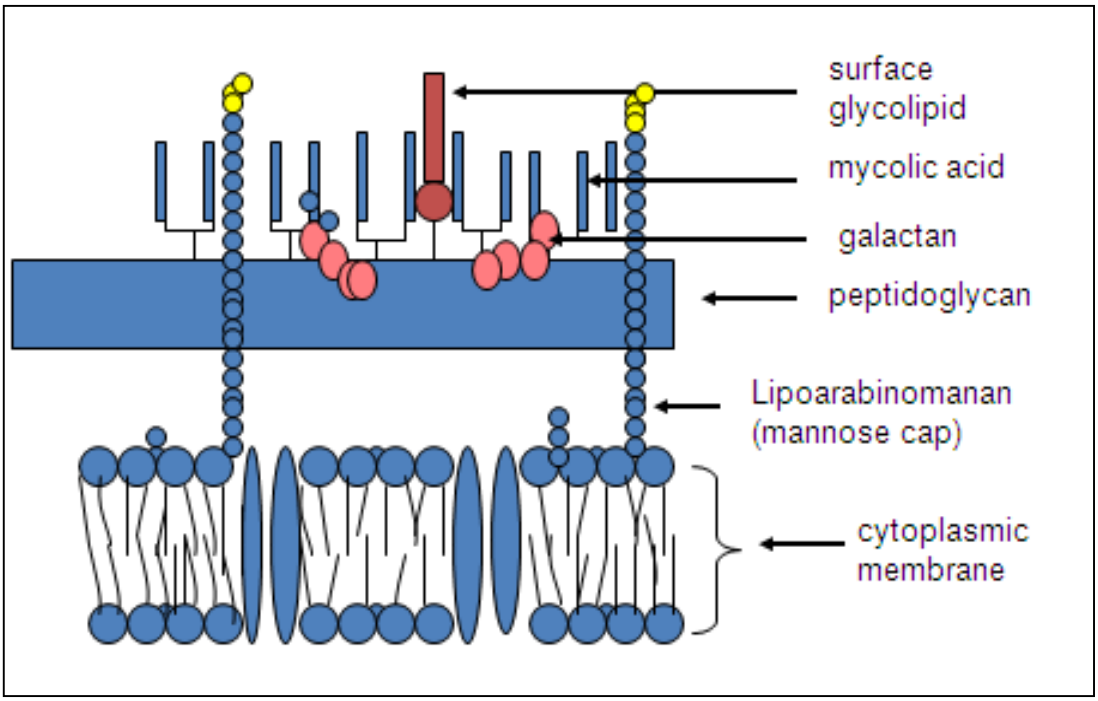


Figure 2.7. A schematic model of the cell envelope of *M. leprae***.** (Vissa and Brennan, 2001**)**

2.7. Growth and Metabolism of *M. leprae*

Little progress has made in understanding the metabolic properties of *M. leprae* due to the lack of suitable media to culture the bacteria which is very essential to study metabolic property of the organism. Although the genus *Mycobacteria* are found in the soil, water and air, the presence of *M. leprae* in these environment is not precisely known. Since *M. leprae* cannot be cultured on artificial media, it is very difficult to investigate if this bacterium inhibit soil, water or air. The presence of *M. leprae* DNA has been found in soil samples in leprosy endemic areas. However, this does not confirm that its replication in these environments. The optimum temperature for *M. leprae* is 30-33ºC which may accounts for *M. leprae* growth in the peripheral nerves. The doubling time for *M. leprae* is thought to be 14 days. Toxin production by *M. leprae* has not been identified (Wheeler and RC., 1988).

It has been reported that many of the pseudogenes of *M. leprae* are in the metabolic pathways. Key metabolic pathways have been lost due to this genomic downsizing (Scollard et al., 2006). Catalase enzyme which breaks down peroxides appears to be absent in *M. leprae.* However, it is not known how *M. leprae* escapes from the toxic oxygen metabolites. *M. leprae* cannot synthesis purines and hence dependent

on the host for purines (Wheeler, 1987, Scollard et al., 2006). *M. leprae* does not have mycobactin, an iron binding compounds and the siderophores, an Iron chelating compounds (Wheeler and RC., 1988). Hence, it is dependent on its host for iron provision. Interestingly, *M. leprae* synthesises its own ATP and it does not depend on its host for ATP (Lee and Colston, 1986).

2.8. *M. leprae* genome

Analysis of a complete genome sequence of *M. leprae* (TN strain) from Tamil Nadu, India has revealed that *M. leprae* has undergone reductive evolution and massive pseudogene formation (Cole et al., 2001). *M. leprae* genome contains 3,268.203 base-pairs (pb) with an average of G + C content of 57.8% in contrast to *M. tuberculosis* whose genome size is 4,411,532bp and G + C content of 65.6% (Table 2.2). The base composition of DNA of *M. leprae* is different from most other *Mycobacteria*. The G+C content of other *Mycobacteria* is 65-70%. Only 49.5 % of *M. leprae* genome contains protein coding genes and the corresponding value for *M. tuberculosis* is 90.8% (Cole et al., 2001). From 50.5% of *M. leprae* genome which does not code for proteins, 27% contains pseudogenes and the remaining 23.5% of the genome seems either regulatory genes or gene remnants mutated beyond recognition (Cole et al., 2001).

From 1,604 protein coding genes of *M. leprae*, 1,439 genes are also shared by *M. tuberculosis*. If all genes in *M. leprae* genome were active 3,000 proteins are expected compared with the 4,000 proteins predicted in *M. tuberculosis*. However, only 239 and 1800 types of soluble proteins were found in *M. leprae* and *M. tuberculosis* respectively which shows the inertness of the pseudogenes (Cole et al., 2001, Monot et al., 2009). Hence, it is suggested that *M. leprae* has lost more than 2000 genes and these genes could include metabolic genes and this might be an explanation for why scientists have failed to grow *M. leprae* in axenic culture.

Since, *M. leprae* had undergone genome decay and contains large number of pseudogenes, high genetic diversity is expected among *M. leprae* strains. However, comparative genomics revealed genetic diversity is exceptionally rare among the strains. The whole genomic sequencing of strain Br4923 from Brazil, Thai53 from Thailand and NHDP63 from USA showed that the gene content is essentially identical to TN strain (Singh and Cole, 2011).

Mycobacterium lepromatosis has been the only other identified pathogen associated with diffuse lepromatous leprosy, also known as Lucio leprosy. *Mycobacterium lepromatosis* was first identified from Mexican patient by Han et al. (2008). Comparative genomic study has shown that *M. leprae* and *M. lepromatosis* are two different but closely related mycobacterial species. It has been speculated that both species derived from a common ancestor that underwent genome downsizing and gene decay (Singh et al., 2015). The divergence time for the two species is estimated to be 14 million years. It was also suggested that both species have continued to lose genes but from different regions of the genome. According to Singh et al. *M. leprae* appears to be more recent. The hemN gene which is present in *M. lepromatosis* but not in *M. leprae* is the only diagnostic method available to differentiate the two species (Singh et al., 2015). The comparative genomic analysis of *M. tuberculosis, M. leprae* and *M. lepromatosis* is presented (Table 2.2).

Table 2.2. Comparative genomic analysis of *M. leprae* genome. *M. lepromatosis* and *M. tuberculosis* (Cole et al., 2001, Monot et al., 2009, Singh and Cole, 2011, Singh et al., 2015).

Features	*M. tuberculosis*	*M. leprae*	*M. lepromatosis*
Genome size (bp)	4,411,532	3,268,203	3,206,741
G +C (%)	65.61	57.79	57.89
Protein coding genes (%)	90.8	49.5	46.01
Protein coding genes (no)	3959	1614	1477
Pseudogenes (no)	6	1306	1334
Gene density (bp/gene)	1,114	2037	?
Average gene length (pb)	1012	1011	?
Average unknown gene length (bp)	653	338	?

2.9. Antigenic components of *M. leprae*

Identification and characterization of *M. leprae* specific antigens is among the major goals to be attained by researchers for accurate and reliable diagnosis of leprosy. *M. leprae* has two groups of antigens. These antigens are classified as protein and non-protein antigens.

2.9.1. The non-protein antigens

The non-protein antigens are composed of lipid or carbohydrate moieties. These are mainly cell wall associated antigens. The non-protein antigens of *M. leprae* further subdivided into phenolic glycolipid (PGL) and lipoarabinomannan (LAM) antigens.

Phenolic glycolipid-1: Phenolic glycolipid-1 (PGL-1) is the abundant surface glycolipids in *M. leprae.* It is located at the outermost surface of *M. leprae* and thus ideally positioned to interact with host cells components (Figure 2.7).

PGL-1 consists of trisaccharide linked to a phenol which in turn linked to tetramethyl mycocerosic acid. The terminal trisaccharide gives antigenic specificity to *M. leprae*. PGL-1 of *M. leprae* binds complement component C3 in serum and mediates phagocytosis by human monocytes (Schlesinger and Horwitz, 1991b). PGL-I has also been implicated in the tropism of *M. leprae* for Schwann cells, through specific binding to laminin, and to play an important role in down-regulation of the inflammatory immune response and inhibition of dendritic cell maturation and activation, thereby facilitating the persistence of *M. leprae* (Spencer and Brennan, 2011).

Lipoarabinomannan: Lipoarabinomannan (LAM**)** is the major component of the cell wall of *M. leprae* (Figure 2.8). Chemically it is inert i.e. it is stable and indigestible. It consists of repeating units of arabinose and mannose attached through phosphatidylinositol moiety to the cytoplasmic membrane. LAM has diverse biological activities such as scavenging potentially cytotoxic oxygen free radicals, inhibit protein kinase C activity and block the transcriptional activation of interferon-gamma inducible genes in human macrophage cells (Spencer and Brennan, 2011). It has been described that LAM inhibits macrophage activation and hence, used as a virulence factor contributing to the persistence of mycobacteria within mononuclear phagocytes (Spencer and Brennan, 2011).

2.9.2. The protein antigens

The protein antigens are mainly display enzymatic properties. They are either cell wall associated, cytoplasmic or secreted antigens. Following the complete sequence of *M. leprae* genome, about 200 recombinant protein antigens have been identified

although none of them have been found to be qualified for early diagnosis of leprosy (Geluk A. et al., 2011, Wiker et al., 2011, Kumar et al., 2014a).

Cytoplasmic proteins: cytoplasmic proteins (10, 18, 65, and 70 KD) are heat shock or stress related proteins. Other cytoplasmic proteins include the 28, 36 and 49 KD proteins. The 428 KD protein is superoxide dismutase enzyme, homologous with a human mitochondrial enzyme (Hunter et al., 1990).

Cell wall and membrane associated proteins: A wide range of important cell wall and cytoplasmic membrane associated proteins in *M. leprae* has been identified by biochemical and recombinant DNA techniques. Among these proteins, the family of 85B complex proteins are the most widely known proteins. It has been suggested that these proteins have enzymatic function in the biosynthesis of mycolic acid which is a major component of the cell wall of mycobacteria (Melancon-Kaplan et al., 1988).

Secreted proteins: The secreted proteins of *M. leprae* include the 25 and 27 KD proteins. The 25 and 27 KD proteins have been identified by their release into culture filtrates from viable mycobacteria. These proteins are assumed to be the first antigens encountered by the immune system and hence have an important role in protecting the *M. leprae* from the host immunity (Wieles et al., 1994, Harboe and Wiker, 1998).

2.10. Pathology

Leprosy affects nerves, skin and eyes. It may also affect other body parts such as mucosa (mouth, nose, and pharynx), testes, kidney, voluntary or smooth muscles, reticulo-endothelial system, and vascular endothelium. The bacillus has low pathogenicity and only a small proportion of infected people develop signs of the disease and this depends on the immune response of the host. In most people the immune system is able to eliminate leprosy during the early infection stage before symptoms develop. After the bacilli get into the body either through the skin or the nasal discharge, they are taken up by histiocytes in the skin and by the Schwann cells in the nerve upon which they usually provoke an inflammatory response of histiocytes and lymphocytes. The mode of the onset of the response is greatly variable. An early lesion may occur as a vague, ill-defined, hypopigmented (in a

dark skin) or erythematous (in a light skin) patch with anaesthesia is called 'indeterminate" leprosy. The disease can also occur with multiple infiltrated patches or just diffuse skin infiltration (Bryceson and Pfaltzgraff, 1990, Kumar and Kumar, 2010).

Most indeterminate lesions will restore spontaneously. However, if the host defence mechanism fails to control the multiplication of the bacilli, then the condition progresses into one of the forms that make up the spectrum of the disease. The clinical form and the subsequent outcome of the disease depends on the nature and extent of the host's immune response (Ridley, 1988).

When the host immunity especially the cell-mediated immunity (CMI) is strong, the clinical form of the diseases is that of tuberculoid leprosy (TT). The disease is localized to one or few sites in the skin and few in the large peripheral nerves. It is characterized by granulomatous inflammation associated with infiltration and destruction of nerve fibres. The lesion is characterized by well-defined margins, which is markedly anaesthetic and do not show the presence of acid-fast bacteria. Lesion infiltrate primarily consists of foci of well-developed epithelioid macrophages, with or without Langhans-type of multi-nucleated giant cells surrounded by a cuff of lymphocytes. (Myrvang et al., 1973, Modlin et al., 1983, Schlesinger and Horwitz, 1991b, Sampaio et al., 2011).

If the host immunity is failed to control the bacilli, the disease progress to the lepromatous leprosy (LL) form. This form of leprosy is marked by numerous bacterial growth both in the skin and in the nerve. In the skin, macrophages fail to differentiate and they laden by the bacilli. Lymphocytes are scanty or absent. Large number of bacilli are found in the Schwann cells of cutaneous nerve fibres. Clinically the disease is characterized by multiples of lesions all over the body which progress to nodules if untreated. It is characterized by symmetrical loss of sensation followed by loss of motor nerves. Antibodies to *M. leprae* are abundant in the sera of these patients but are ineffective in controlling the progress of the disease (Ridley, 1988, Walker and Lockwood, 2006).

In-between the two polar leprosy forms are the immunologically unstable borderline forms showing clinical and histopathological characteristics

intermediate to the polar forms. The formation of small granulomas is typical feature of borderline forms. Within these groups, there is a gradual decrease in cell-mediated immunity from BT to BL and this is inversely correlated with the bacillary load (BI) within the lesions(Ridley and Jopling, 1966, Godal and Negassi, 1973b).

2.1. Genetic Susceptibility

Genetic studies provides evidence that genetic variation in human populations contributes to susceptibility to infectious disease. The genetic susceptibility is thought to be of importance not only in predisposing or protection against developing clinical disease, but also in determining the clinical futures of the disease in individuals. It has been shown that the host genetic factors influence the acquisition of leprosy and the subsequent clinical course of the disease (Bhat and Prakash, 2012).

In a linkage study included 197 Vietnamese leprosy families and 587 Brazilian leprosy cases identified the first major polymorphic risk factors in the shared promoter region of PARK2 and PACRG (Mira et al 2004). Single-nucleotide polymorphism (SNP) association studies in two Vietnamese familial samples involved 298 cases showed a low producing lymphotoxin-*a* (LTA)+80A allele was significantly associated with increased risk of leprosy (Alcais et al., 2007).

A genome-wide association study included 706 leprosy patients and 1225 healthy controls of the Han Chinese family showed variants of genes in the NOD2-mediated signalling pathway (which regulates the innate immune response) associated with susceptibility to infection with *M. leprae* (Zhang et al., 2009).

An Indian study has reported that the human histocompatibility leukocyte antigen (HLA)-DR2 was found to be associated with susceptibility to TT compared to LL showing the importance of HLA in the leprosy spectrum (van Eden et al., 1980). A case -control study which involved 32 LL patients with 32 endemic controls in Venezuela has shown that the frequency of HLA specificity LB-E12 (MB1, DC1 and MT1) was significantly increased in lepromatous leprosy patients compared to the endemic controls (Ottenhoff et al., 1984). Similarly, in a study of the relation between HLA and lepromatous leprosy, the analysis of HLA haplotype segregation in 28 families from Venezuela with multiple cases of different types of leprosy has

shown that HLA-DR3 was inherited preferentially by children with polar tuberculoid leprosy while, HLA-MT1 was inherited preferentially by children with lepromatous leprosy (van Eden et al., 1985). HLA class I HLA-A*1102-B*4006-Cw*1502 haplotype has been reported showing a highly significant association with leprosy susceptibility in Indian patients (Shankarkumar et al., 2003). Among the class II genes the HLA-DRB1 locus specifically DRB1*15 and DRB1*16 are associated with leprosy susceptibility in India, Thailand and Brazil. The HLA-DQw1 locus was found to be associated with LL patients (Correa Rda et al., 2012)The vitamin D receptor (VDR) has been shown associated with leprosy in an Indian population where a T-C substitution at codon 352 at the 3' gene region resulted in susceptibility to lepromatous leprosy in the presence of genotypes TT and tuberculoid leprosy if the genotypes is CC (Roy et al., 1999). Other SNPs to be associated with disease and/or the development of reactions in several genes, such as TNF-α, IL-10, IFN-γ, HLA genes, and TLR1 are also suggested (Fitness et al., 2002).

2.12. Clinical Features of leprosy

The clinical features of leprosy vary depending on the host repose to *M. leprae*. Leprosy patients usually present with skin lesions, numbness or weakness caused by peripheral nerve involvement or more rarely a painless burn or ulcer in an anaesthetic hand or foot. A leprosy reaction may be a presenting feature of the disease (Walker and Lockwood, 2006).

2.12.1. Cutaneous features

Early skin lesions may be rather poorly defined hypo-pigmented or erythematous macules. Sensation in these early stages may be unaltered. Tuberculoid (TT) leprosy is characterized by a single or few lesions with defined edges. The lesions can either macules or plaques. In dark skin hypopigmentation predominates over the erythema or copper colour more usually seen in lighter skin. The lesions are mostly anaesthetic and involve alopecia (hair loss). Anaesthesia may not be present in facial lesions. Lesions can be dry and scaly, hypohidrotic (diminished sweating ability), and hairless. Spontaneous resolution can occur in a few years. Progression can also occur, leading to borderline-type leprosy (Walker and Lockwood, 2006).

Patients with borderline tuberculoid (BT) have similar lesion forms to those with TT leprosy. However, the lesions in BT patients are smaller and tend to be more numerous with less demarcated edges. The skin lesions in patients with borderline-borderline (BB) can be macular or papular or plaque-like or even the combination. Cutaneous lesions consist of numerous, irregularly shaped plaques that are less well defined than those in TT or BT. The lesions are moderately anaesthetic (Guinto et al., 1983, Walker and Lockwood, 2006).

In borderline lepromatous leprosy (BL) lesions are numerous and widespread bilaterally. The surface of the lesions are mostly smooth and shiny with ill-defined edges. Lepromatous leprosy (LL) is characterized by numerous skin lesions that are widespread and symmetrical distribution. Early cutaneous lesions consist mainly of pale macules with ill-defined edges. Papular and nodular lesions are seen in advanced disease. Early lepromatous leprosy lesions have little or no loss of sensation (Guinto et al., 1983, Walker and Lockwood, 2006).

2.12.2. Neural features

The neural involvement of leprosy includes damage to the nerve trunks as well as to the cutaneous nerve endings. Nerve involvement in leprosy affects sensory, motor and autonomic function of peripheral nerves. Sensory clinical presentations are the most common and usually open the clinical picture. Leprosy reactions can cause further nerve damage. The nerve damage due to leprosy leads to disability and deformity such as clawing of the hand. Deformity also occurs through impaired sensation leading to trauma and secondary infection which causes tissue damage (Grimaud and Vallat, 2003, Nascimento, 2013).

In TT leprosy, the nerve thickening is usually confined to one nerve and the damage to peripheral nerves is limited. In LL the increases in nerve size is symmetrical while the nerve enlargement is frequently segmental rather than diffuse and uniform. The destruction of dermal nerves leads to a glove and stocking pattern of sensory loss.

Pure neuritic leprosy (PNL) is characterized by neural impairment (neuropathy) without evidence or history of typical skin lesions. It manifests as enlargement of one or more peripheral nerves, with or without tenderness, sensory motor

impairment, and muscular atrophy, particularly of the hand and foot muscles (Payne et al., 2015). Pure neuritic leprosy accounts for about 4% cases of leprosy in India (Kumar et al 2004) while it was 0.5% in in an Ethiopian cohort (Saunderson et al., 2000b).

2.12.3. Other features

Other features of leprosy includes saddle nose deformity; visual loss due to lagophthalmos (an inability to close the eyes completely), corneal ulceration, acute or chronic iridocyclitis and secondary cataract, testicular atrophy and facial palsy. The involvement of nasal mucosa in lepromatous leprosy gives rise to nasal stuffiness and epistaxis in advanced cases which eventually lead to saddle nose deformity. Nasal deformity contributes significantly to the stigma associated with leprosy (Rafferty, 2005).

2.13. Classification

The clinical and histological features of leprosy is correlated with the immunological state of the host which determines prognosis and constitutes the basis for the classification of this disease. There are two approaches to classifying leprosy patients: The Ridley-Jopling (RJ) and WHO classification systems. The Ridley-Jopling system mainly is used for classification of leprosy for scientific purposes and the WHO system is used for operational activities (Figure 2.9).

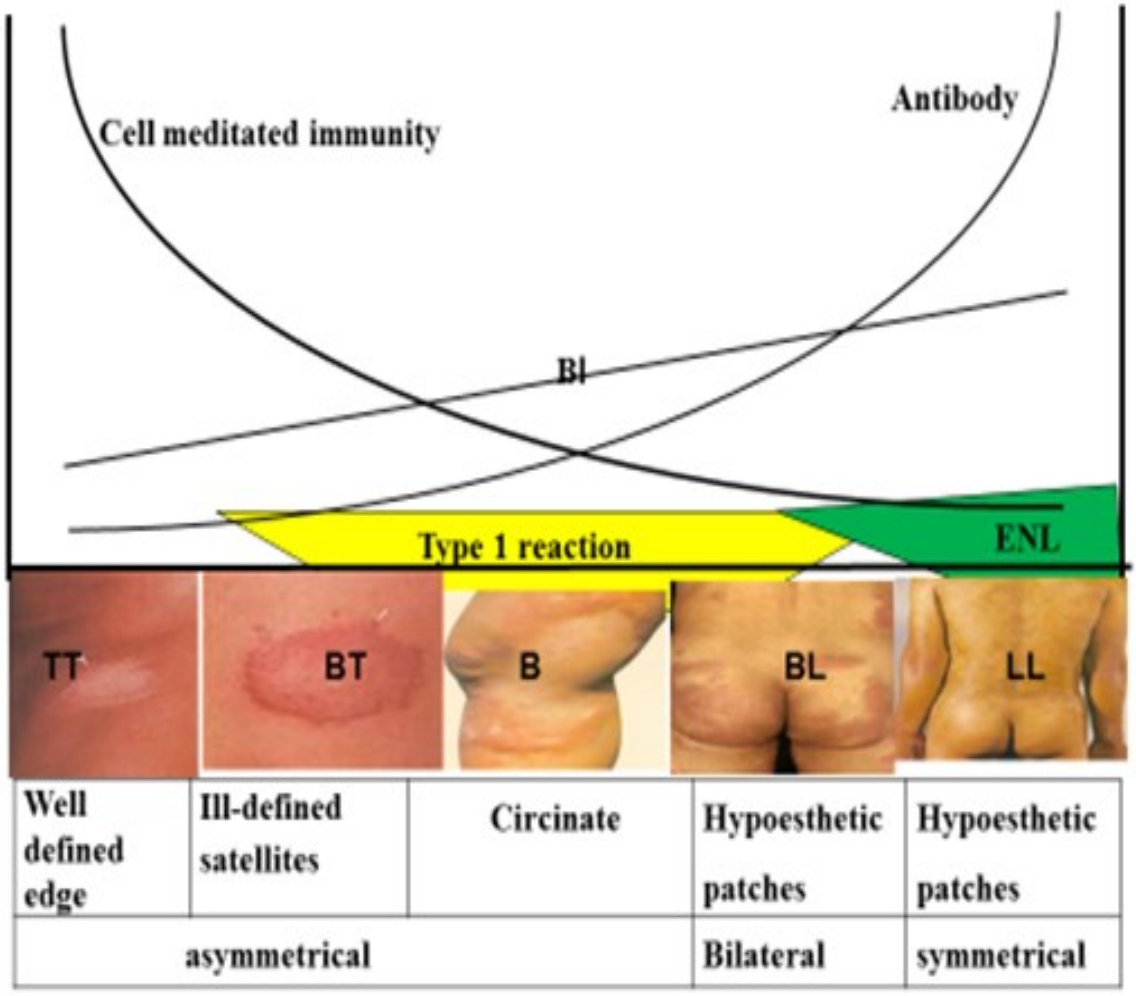
2.13.1. The Ridley-Jopling classification

The bases for the Ridley-Jopling classification system are clinical, histological features and bacteriological index. According to this classification scheme, there are two poles of spectrum and three borderline forms of leprosy. The two poles of the spectrum are lepromatous leprosy and tuberculoid leprosy. The borderlines are designated as borderline tuberculoid (BT), mid borderline (BB) and borderline lepromatous (BL) (Ridley and Ridley, 1983).

Patients with LL have multiple, symmetrically distributed lesions throughout the body (skin, nerves, eyes and nasal mucosa). Histopathologically, LL lesions show an infiltrate mainly composed of macrophages, showing varying degrees of foamy changes, and few, scattered lymphocytes, predominantly of the $CD8^+$ T-cell subset (Walker and Lockwood, 2006). Patients with lepromatous leprosy have high bacterial index. Anti-*M. leprae* antibody titres are high in the sera of LL patients but do not control the multiplication of the bacilli.

Patients with tuberculoid leprosy (TT) have few lesions with well-defined margins, which are markedly anaesthetic and do not show the presence of acid-fast bacteria. Lesional infiltrate primarily consists of foci of well-developed epithelioid macrophages, with or without Langhans' type of multi-nucleated giant cells surrounded by a cuff of lymphocytes. The T-lymphocyte subsets are predominantly of the $CD4^+$ type (Myrvang et al., 1973, Modlin et al., 1983, Sampaio et al., 2011).

In-between the two polar leprosy forms are the immunologically unstable borderline forms including borderline lepromatous (BL), mid-borderline (BB) and borderline tuberculoid (BT), showing clinical and histopathological characteristics intermediate to the polar forms. Within these groups, there is a gradual decrease in cell-mediated immunity (CMI) from BT to BL and this is inversely correlated with the bacillary load (BI) within the lesions (Figure 2.8) (Ridley and Jopling, 1966, Godal and Negassi, 1973b).



BI= Bacillary Index

Figure 2.8. Ridley and Jopling leprosy classification schemes (Lockwood, 2004)

2.13.2. The WHO classification

The WHO recommended an operational classification to serve as a basis for chemotherapy to be used in the field when slit skin smears are not available. The WHO classification is based on the number of skin lesions. Patients with 1 to 5 skin lesion are classified as paucibacillary (PB) and those with 6 or more skin lesion are grouped as multibacillary (MB) type.

A comparative study from Philippines has reported that about 38-51% of leprosy patients were misclassified as PB by WHO classification criteria when 264 leprosy cases had been re-classified using the standard histopathological and microbiological criteria (Pardillo et al., 2007). Similarly, of 60 PB and 40 MB new untreated leprosy patients by WHO classification scheme in India, 19 (37%) of them were misclassified as PB and 7 (15%) of them as MB when correctly classified using the histology and slit skin smear microscopy (Gupta et al., 2012). The INFIR cohort study of Indian patients has shown that MB cases are very heterogeneous and encompasses patients with no detectable bacteria and high immunological activity through to patients with high bacterial loads (Lockwood et al., 2012). Thus, WHO classification schemes may be a risk for under-treatment of MB cases.

2.14. Diagnosis of leprosy

The diagnosis of leprosy is mainly based on clinical features. Silt skin smear (SSS) test and histological appearances are frequently used to support the clinical diagnosis. Other methods such as serological testing and DNA based PCR detection of *M. leprae* are sometimes used to supplement the clinical findings.

The Cardinal Signs of Leprosy

Three cardinal signs have identified for the clinical diagnosis of leprosy.

1. Skin lesions with definite sensory loss. The lesion could be raised or flat, light or pigmented

2. Thickened or enlarged peripheral nerve(s) with loss of sensation and /or weakness of the muscles supplied by that nerve

3. Presence of acid-fast bacilli (AFB) in a slit skin smear or tissue biopsy

Any one of these signs has been regarded as sufficient for the diagnosis of leprosy (World Health organization, 1998).

Slit skin smear test (SSS): The slit skin smear test is the most simple and frequently used method to classify leprosy cases into paucibacillary and multibacillary cases. . An incision of the skin (5mm log and 2mm deep) is obtained from multiple sites and stained by the Ziehl-Neelsen method also called Acid Fast Bacilli stain (AFB). The AFB stained slides are used for determining the bacillary index (BI). BI is a logarithmic scale (1 -6) quantifying the density of *M.leprae* on a silt skin smear under the microscope.

From a cross-sectional study in India, slit skin smear test confirmed the presence of AFB in 59.8% (64/107) of MB and only in 1.8% (1/57) of PB cases (Banerjee et al., 2011). Although this method has high specificity, its sensitivity is as low as less than 10% for PB patients since in such patients the bacilli load is very low to be detected by this method (Santos et al., 2013).

Skin Biopsy: Histopathological examination of tissue sections from suspected lesions is the gold standard for the diagnosis of leprosy. The use of skin biopsy supports the clinical findings and used as confirmatory diagnosis of leprosy. However, the procedure demands medical facilities and a histopathologist which limits its application in many countries where leprosy is endemic.

***M. leprae* specific antigen (PGL-1):** The use of serological assays based on the employment of *M. leprae* specific antigens, mainly phenolic glycolipid (PGL-1) has opened new methods of monitoring *M. leprae* infections, since antibody levels

are associated with intensity of exposure and systemic involvement to the bacteria. However, serological tests do not detect all clinical infections, since paucibacillary patients do not develop significant levels of antibody response. Many studies have demonstrated that multibacillary patients have high titers of *M. Leprae* specific antibodies but paucibacillary patients have low or absent titters. The antibody response to PGL-1 is primarily IgM and its level gradually rises from TT to LL indicating its direct correlation with bacilli index (BI). It was reported that 90 to 95% multibacillary and 20-40% paucibacillary leprosy patients have a positive antibody in a PGL-1 based test (Geluk A. et al., 2011). It has also been shown that the false positivity rate of PGL-1 based tests is greater than 10% in leprosy endemic area (Duthie et al., 2007). Consequently, PGL-1 based test has not been widely implemented in field situations as screening tools.

ML-Flow test: The ML- Flow test is a simple and rapid immunochromatographic flow test used for the detection of IgM to PGL-I. It is a simple dipstick assay which can be used at field level using whole blood sample. The ML-Flow test has been reported to be comparable to the ELISA in its sensitivity (97.4%), being able to detect more than 90% of MB patients and 40% of PB patients, with background seropositivity in endemic controls at around 10%. Moreover, this dipstick test was reported to be applicable in the field, identifying multibacillary patients without the need for a slit-skin smear test (Buhrer-Sekula et al., 2003). However, its low sensitivity to PB patients hindered the use of this test as leprosy diagnostic test in the leprosy endemic countries.

LID-1 test: LID-1 (leprosy Infections Disease Research Institute diagnostic 1) is a chimeric fusion of two proteins (ML0405 and ML2331) which is used for leprosy diagnosis on the basis of immunoglobulin G (IgG) reactivity. Positive titres of antibodies against LID-1 protein were found in 87-92% MB and 7-48% PB patients in different populations of Brazilian leprosy patients (de Souza et al., 2016). It has been reported that some household contacts presenting with high titres of antibodies against LID-1 were developed clinical symptoms of leprosy after one year suggesting a role for this protein in the monitoring of contacts (Duthie et al., 2007).

NDO-LID® rapid test (Orange Life®, Rio de Janeiro, Brazil) was developed by impregnating nitrocellulose membranes with ND-O-LID-1, a conjugation of the ND-O (a synthetic mimetic of PGL-I disaccharide) and the LID-1 protein (Duthie et al., 2014). In a study conducted among Brazilian leprosy patients, it was found that NDO-LID detected 87.0% MB patients and only 20.2% PB patients (Paula Vaz Cardoso et al., 2013).

UCP-LFA test: The up-converting phosphor (UCP)-Lateral flow assay is a combination of UCP reported technology with lateral flow test which is used for detection of a variety of analytes such as drugs of abuse, protein and polysaccharide antigens from pathogens, bacterial and viral nucleic acids and antibodies against several diseases. The phosphorescent reporter utilized in UCP-LFAs is excited with infrared light to generate visible light, a process called up-conversion. UCP-based assays are highly sensitive since up-conversion does not occur in nature, avoiding interference by autofluorescence of other assay components. Importantly, UCP- LF test strips can be stored as permanent records allowing re-analysis in a reference laboratory (Bobosha et al., 2014a). In UCP-LF assay can be developed for a single cytokine or antibody test or for combination of cytokines or antibodies termed as a multiplex UCP-LFA. Previous studies have shown that INF-Ɣ-UCP-LFA and IP-10-UCP-LFA can be used to discriminate leprosy infected individuals from endemic controls in Ethiopian population (Bobosha et al., 2014a). Corstjens et al. (2016), have shown by using a UCP-LFA, that IP-10 was significantly increased during the onset of T1R reaction than before the onset of reaction and was significantly reduced after treatment. Therefore, unlike the other leprosy diagnostic tests, UCP-LFAs may be used for leprosy diagnosis as well as for monitoring multiple immune responses markers during the onset and treatment of leprosy reactions.

Molecular based diagnosis: New molecular biology methods have been developed as reliable and sensitive diagnostic tools for the identification of pathogens in many infectious diseases. The detection of the DNA of the infectious microbe by polymerase chain reaction (PCR) is the most widely used method. Following the *M. leprae* genomic sequence many scientists tried to adopt the experience of PCR based diagnosis of infectious diseases to leprosy.

According to the reports of Martinez et al., a detection rate of 100% among multibacillary patients and 62.5% to 79.2% among paucibacillary (PB) patients has been attained using both conventional and real time PCR from 69 biopsy samples (25 LL, 11 BL, 3 BB, 24 BT and 6 healthy controls) from Brazil (Martinez et al., 2006). These authors reported that the clinical sensitivity of real time PCR and conventional PCR was 91.3% and 82.6% respectively. An Indian study has shown that 85.9% MB and 75.5% PB patients were successfully diagnosed by PCR (Banerjee et al., 2011). Hence, although PCR increases the sensitivity of *M. leprae* DNA detection in patient sample over conventional methods, satisfactory result has not been achieved with regard to the detection of PB cases (Kurosaki et al., 2015).

2.15. Diagnosis and grading disability

The WHO leprosy disability grading system grades leprosy patients based on the disabilities of eyes, hands and feet (Table 2.3). The highest grade of disability of any of these body sites is used as an overall indicator of the disability status of a person with leprosy (Alberts et al., 2011).

Table 2.3. WHO leprosy disability classification (Alberts et al., 2011).

Body sites	Grade	Criteria
Hands and Feet	0	No anaesthesia, no visible deformity or damage
	1	Anaesthesia present, but no visible deformity or damage
	2	Visible deformity or damage present
Eyes	0	No eye problem due to leprosy; no evidence of visual loss
	1	Eye problems due to leprosy present, but vision not severely affected as a result (vision: 6/60 or better; can count fingers at 6 metres).
	2	Severe visual impairment (vision worse than 6/60; inability to count fingers at 6 metres); also includes lagophthalmos, iridocyclitis and corneal opacities.

Among the newly diagnosed Indian INFIR cohort study, 40.9% of them had WHO disability grade-1 and 9.6% grade-2 at enrolment (Van Brakel et al., 2005b). The Bangladesh Acute Nerve Damage Study (BANDS) cohort had a prevalence of 9.6% grade-1 and 6.0% grade-2 disability in 2,664 leprosy patients at enrolment (Croft et al., 1999). However, the rate of grade-1 disability was 28.5% and that of grade-2 was 18.2% in MB patients. A survey of 1,358 Indonesian leprosy patients, who had been released from MDT up to 5 years earlier, found that 75% of the patients had physical impairment (van Brakel et al., 2012).

In Brazil about 6.5% of the new cases reported to WHO in 2014 had grade-2 disabilities at presentation. The rate of grade two disability in new cases of Ethiopian leprosy patients reported to WHO in 2014 was 10.2% (WHO, 2015). According to WHO 2014 epidemiological data, 6 countries reported the highest rate of grade-2 disability in newly diagnosed leprosy cases. These countries include: Chad (25.2%), Burkina Faso (23.6%), China (20.0%), Pakistan (17.6%) and Tanzania (17.0%) indicating the delay of diagnosis and treatment in these countries (WHO, 2015)

2.16. Differential Diagnosis

The differential diagnosis of leprosy is broad due to the variability of clinical manifestations of leprosy. Patients may have skin lesions occurred by fixed drug eruption, morphea (localised scleroderma), dermatophytosis, dermal filariasis, eczema, scars, nodular cutaneous leishmaniasis, post-kala azar dermatitis or keloids.

2.17. Treatment of leprosy

Leprosy is curable if treated with the correct regimen. A successful treatment for leprosy was discovered in 1947 with dapsone which remains a component of present day multi-drug combination therapy (MDT) (Table 2.4).

The WHO recommends that patients diagnosed as having leprosy should receive a multi-drug combination therapy. Multi-drug combination therapy was introduced in 1982 following the emergence of resistance to dapsone-only regimen (WHO, 1982). The first-line agents are rifampicin, clofazimine and dapsone. Since 1995, WHO provides free MDT for all patients in the world provided by the Novartis

Foundation for Sustainable Development. Paucibacillary patients are treated with rifampicin and dapsone for six months and the recommendation for individuals with MB leprosy cases is three drugs: rifampicin, dapsone and Clofazimine for 12 months (Table 2.6). Between 1985 and 2014, about 16 million individuals received MDT (WHO, 2015).

Rifampicin

Rifampicin is a bactericidal antibiotic used to treat *Mycobacterium* infections such as tuberculosis and leprosy. Previous studies in untreated multibacillary leprosy patients have shown rifampicin administered in single doses of 600mg killed more than 99% of *M. leprae* within four days (Levy et al., 1976). The mechanism of action of rifampicin is through inhibition of DNA-dependent RNA polymerase and hence, impairs the bacterial DNA transcription (Wehrli, 1983). Resistance to rifampicin has been shown due to clustered mutations in *rpoB* genes of clinical isolates of *M. leprae* (Nakata et al., 2012).

Clofazimine

Clofazimine has been used in the treatment of multibacillary leprosy since 1962. It is a bactericidal drug with both antibacterial and anti-inflammatory activity. The anti-inflammatory activity of clofazimine is through its immunosuppressive effects. It works through binding to the guanine bases of bacterial DNA, thereby blocking the template function of the DNA and inhibiting bacterial proliferation. It also increases activity of bacterial phospholipase A2, leading to release and accumulation of lysophospholipids, which are toxic and inhibit bacterial proliferation. The lipophilic property of clofazimine enables it to accumulate in skin and nerves, while its anti-inflammatory activities are potentially useful in controlling leprosy reactions (Cholo et al., 2012). The side effects of clofazimine includes increased skin pigmentation, and dryness, which occur as the drug becomes clinically effective (Ramu and Iyer, 1976).

Dapsone

Dapsone is a sulphur compound with bacteriostatic action, used in the treatment of leprosy. Its mode of action is via inhibition of the synthesis of dihydrofolic acid,

(Vitamin B9), a precursor of tetrahydrofolic acid which serves as a cofactor in the metabolism of amino acids and nucleic acids. By 1951, oral dapsone (100mg daily dose) was introduced as the standard treatment of leprosy. However, in the late 1960s relapse in patients who had previously treated with dapsone and resistance in patients who had never been exposed to dapsone was reported. Haemolytic anaemia and skin reactions are among the few reported side effects of dapsone (Deps et al., 2012).

Table 2.4. World Health Organization recommended MDT regimens for leprosy treatment

Type of Leprosy	Drug treatment		Duration of treatment
	Monthly supervised	Daily self-administration	
Paucibacillary	Rifampicin 600mg	Dapsone 100mg	6 months
Multibacillary	Rifampicin 600mg	Dapsone 100mg	12 months
	Clofazimine 300mg	Clofazimine 300mg	

2.18. Relapse in leprosy and drug resistance

Relapse in leprosy is defined as " A patient who successfully completes an adequate course of MDT, but who subsequently develops new signs and symptoms of the disease either during the surveillance period (2 years for PB and 5 years for MB leprosy) or thereafter" (World Health organization, 1998). Relapse is diagnosed by the appearance of definite new skin lesions and/or an increase in bacteriological index (BI) of two or more units at any single site compared to BI taken from the same site at previous examination.

The relapse rates following MDT are low. WHO estimated a risk of relapse of 0.7% for MB and 1.07% for PB patients 9 years after stopping MDT (World Health Organization, 2006). A retrospective study included 3,248 leprosy patients who completed the WHO-MDT during the period of 1987-2003, in Tamil Nadu, had reported an overall relapse rates of 0.84% in MB and 1.9% in PB leprosy (Ali et al.,

2005). Relapses cases were not found in 502 patients who completed MDT in the AMFES (ALERT MDT Field Evaluation Study) cohort, 8 years after completion of treatment (Saunderson et al., 2000b). In a long- term follow-up study of up to 16 years in the Philippines, the relapse rate amongst MB patients was 6.6%) (Balagon et al., 2009). Similarly a relapse rate of 1.97% in MB cases and 1.3% in PB was reported from Agra field based cohort study in India (Kumar et al., 2013). A retrospective cohort study of 11 years in Colombia, had reported a relapse rate of 20.0% in 165 leprosy patients (Guerrero-guerrero et al., 2012).

Genetic studies have identified mutations within drug target genes in *M. leprae*, which confer resistance to rifampicin, clofazimine and dapsone. Studies have shown that drug resistance in *M. leprae* is occurred through gene mutations encoding drug targets. These mutations occur spontaneously as a result of errors in DNA replication and they can be enhanced in a population of susceptible to *M. leprae* by inappropriate drug therapy. Drug resistant *M. leprae* mutants can be obtained during initial infection from the infection sources (primary drug resistance) or from inadequate treatment (secondary drug resistance) (Williams and Gillis, 2012, Mejí et al., 2014).

2. 19. Immunology of leprosy

Intracellular pathogens are a major cause of global morbidity and mortality. These pathogens include different species such as *Mycobacteria*, *Leishmania*, *Listeria*, *Salmonella*, *Chlamydia*, *Rickettsia, Trypanosoma* and others. Intracellular pathogens live inside the host cells where they are protected from the effector cells of the immune system. These pathogens prefer the mononuclear phagocytes of the host cells, which are also an important component of the innate immune system. In principle, the phagocytes have to restrict the intracellular multiplication of the pathogen by phagosome lysosome fusion, generation of reactive oxygen and nitrogen intermediates or restricting nutrient availability to the pathogen. They also produce soluble mediators such as cytokines and chemokines to increase the trafficking of T- and B- lymphocytes to the site of infection. Furthermore, the phagocytes along with other specialized antigen-presenting cells guarantee the protein and lipid antigens from the pathogens are processed and presented to the host T-lymphocytes for further action (Thi et al., 2012, Ribet and Cossart, 2015).

Given that these innate immune effector cells are normally programmed to destroy ingested pathogens and promote the development of adaptive immunity, these pathogens may manipulate the host innate defence mechanism to survive and replicate (Figure 2.9) which ultimately leads to a state of disease (Diacovich and Gorvel, 2010).

It is thought that about 99% of individuals infected by *M. leprae* do not develop leprosy (Singh et al., 2014). This indicates the capacity of the host immune response in controlling the disease progression although much of the pathology of leprosy is thought to be related to an inappropriate immune response especially in reactional sates (Godal and Negassi, 1973a, Baumgart et al., 1993). However, factors which regulate the intensity of immune response in leprosy is yet to be investigated. The role of various immune cells and their soluble produces in regulation of immune response in leprosy has been the current focus of intense investigation and the next section reviews some of the new advances in this area.

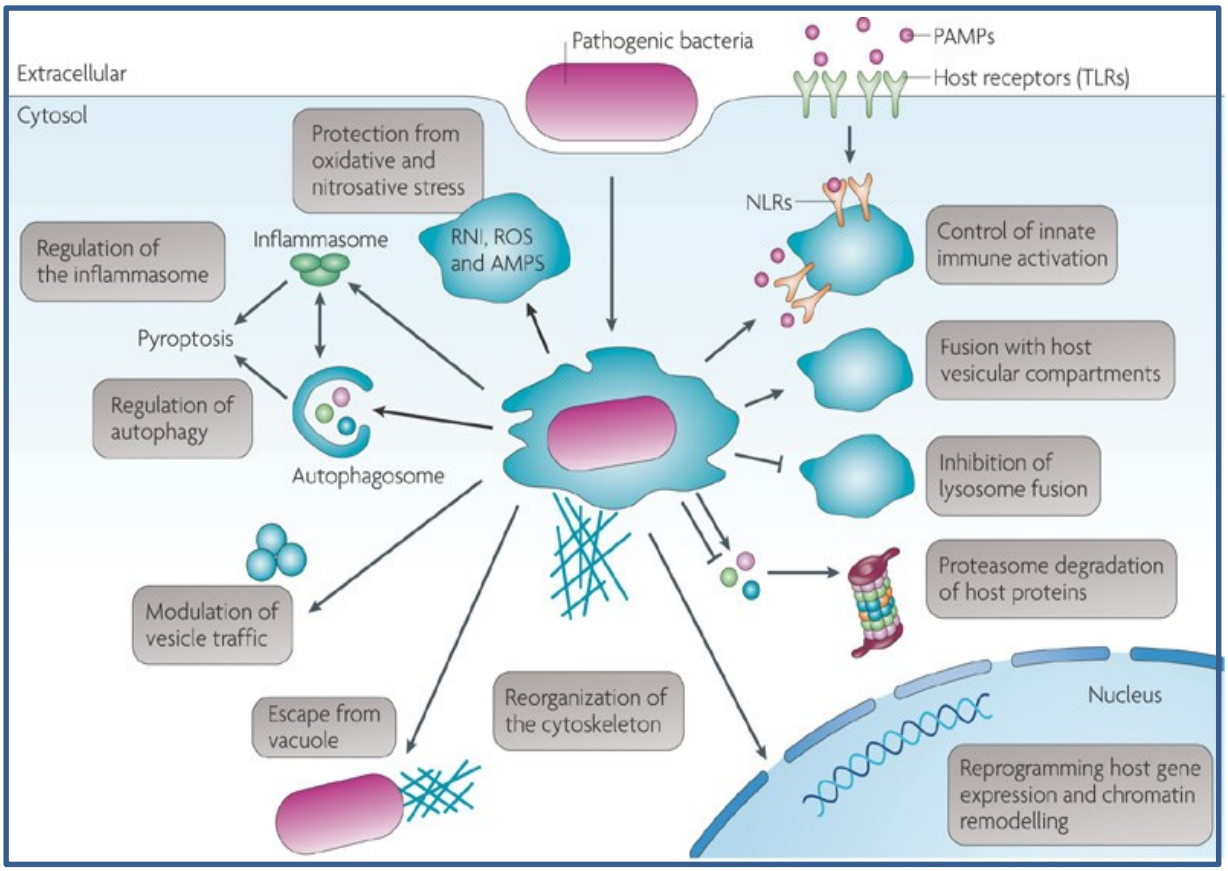


Figure 2.9. Manipulation of host innate immunity by intracellular pathogenic bacteria. (Diacovich and Gorvel, 2010).

2.19.1. Innate Immunity and Leprosy

The innate immune response is comprised of mainly of phagocytic cells and is capable of immediately recognizing and responding to microbial invasion. Macrophages and neutrophils are professional phagocytic cells acting as effector cells to resolve the infection. Dendritic cells (the professional antigen-presenting cells) and macrophages are also vital for the initiation of adaptive immunity and the subsequent generation of specific immunity.

2.19.1.1. Toll like receptors

The innate immune system is capable of recognizing a wide spectrum of pathogens through various receptors that bind highly conserved structures on the microbial components. Cells of the innate immune system are equipped with germline encoded pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs), which are shared among groups of pathogens (Modlin, 2010). The family of toll like receptors (TLRs) is the major and most extensively studied class of PRRs. TLRs are thought to play a crucial role in the recognition of microbes and subsequent induction of the immune responses. These receptors are expressed on immune cells, such as monocytes, macrophages, dendritic cells (DC), and granulocytes, and at sites of host-pathogen interaction such as airway epithelium and skin (McInturff et al., 2005). Signalling through TLRs involves myeloid differentiation 88 (myD88), interleukine-1 receptor-associated kinase (IRAK), and TNF-receptor associated factor (TRAF), followed by activation of nuclear factor (NF)-kB (McInturff et al., 2005).

Activation of TLRs results in upregulation of the production of inflammatory mediators such as TNF-α, IL-6 and IL-12 and microbial killing mechanisms like nitric oxide (NO) production (Dearman et al., 2009). Toll-like receptor 2 (TLR2) is critical in the immune response to mycobacterial infections and the mutations in the TLR2 have been shown to confer the susceptibility to severe infection with mycobacteria. TLR2 is activated by bacterial lipoproteins including mycobacterial antigens (Kang and Chae, 2001). Initially it has been shown that TLR2 SNP was associated with susceptibility to lepromatous leprosy in Korean population (Kang et al., 2002). However, a study involving 286 Indian leprosy patients and 183

ethnically matched controls, has identified the SNP as a pseudogene and does not encode a TRL2 gene (Malhotra et al., 2005).

It has been described that TLR1 1602S SNP was associated with decreased risk of developing leprosy (Johnson et al., 2007). The association of TLR1 248SS and protection against leprosy has been described by (Schuring et al., 2009). Hence, a polymorphism that would reduce TLR2/1 signalling would protect against leprosy. In a study which included 842 patients with leprosy in Bangladesh, has shown that patients who experienced reversal reactions and/or erythema nodosum leprosum reactions during the 4-year follow-up had TLR1 N248S-linked trait suggesting that the TLR1 N248S SNP may affect the progression from infection to disease as well as the disease course and the risk of developing reactional episodes in this population (Schuring et al., 2009). Recently, a study in a Brazilian leprosy patients has reported that TLR1 N248S SNP is associated with leprosy risk and regulates immune activation during mycobacterial infection (Marques Cde et al., 2013).

An Ethiopian cohort study which included 441 patients with leprosy and 193 healthy controls at ALERT hospital has shown that two SNPs in TLR4 (896G →A [D299G] and 1196C →T [T399I]) are associated with protection against leprosy (Bochud et al., 2009). These authors have also reported that *M. leprae* downregulates TLR4-mediated cytokine production in monocytes. Conversely, a case-control study which included 270 Malawi patients with leprosy cases and 490 healthy controls has shown that having the polymorphism TLR4 D299G does not provide protection against leprosy (Fitness et al., 2004) .

The differing and sometimes conflicting results of genetic studies may be attributed to differences in study design and sample size. It is also possible that different populations have distinct genetic susceptibilities (Campbell and Tishkoff, 2008).

2.19.1.2. Macrophages

The macrophage is the major host cell of *M. leprae* bacilli. Schwann cells and keratinocytes are the others cells of the host which harbour *M. leprae* (Kumar et al., 2014a). Regardless of the efficient bactericidal mechanisms of macrophages, *M. leprae* may survive in these cells in two ways. Firstly, *M. leprae* inhibit phagosome lysosome fusion and eventually escape from the phagosome and enter

the macrophage cytoplasm. Secondly, perhaps due to the failure of macrophage activation during the uptake of bacilli. This is further explained by the fact that uptake of *M. leprae* into the macrophages is selectively mediated by the complement receptors, CR1 and CR3, which bind to the surface of the bacteria by PGL-1 and hence inhibits oxidative burst. PGL-1 has been shown to scavenge oxygen radical thereby, *M. leprae* resist the intracellular digestion (Launois et al., 1989, Schlesinger and Horwitz, 1991b).

Lepromatous leprosy is characterized by the accumulation of macrophages which are unable to restrict the multiplication of *M. leprae*. The macrophages from these LL patients failed to activate when stimulated with IFN-γ *in vitro* showing that these macrophages are defective and unable to re-store the normal function. On the other hand, within the TT lesion, bacteria are seen rarely in the macrophages which implies that the macrophages in patients with TT are capable of controlling the *M. leprae* infection (Parmaswaran et al., 1976, Desai et al., 1989). Previous studies has shown that macrophages from LL lesions expressed high level of DC-SIGN which was thought to be associated with a Th2 environment in these lesions (Soilleux et al., 2006). On the other hand, macrophages obtained from patients with TT expressed higher level of toll like receptors-1 (TLR1) and 2 (TLR2) compared to patients with LL. (Krutzik et al., 2003). Hence, it appears that macrophages in LL lesions show a progressive reduction of Th1 activation resulting in a state of antigen specific tolerance. Furthermore, macrophages in LL lesions may down-regulate cellular immunity by reducing antigen-presenting function and secreting Th2 cytokines or by secreting other suppressive factors, such as IL-10 and prostaglandin E2 (PGE-2) (Misra et al., 1995). PGE-2 controls chemokine production, inhibiting the attraction of pro-inflammatory cells while enhancing local accumulation of regulatory T-cells. It also supports activation of dendritic cells but suppresses their ability to attract naive, memory, and effector T-cells (Kalinski, 2012). Attempts to induce macrophage activation in LL patients by local administration of antigens or IFN-γ have not been yet successful (Kaplan et al., 1987b).

2.19.1.3. Neutrophils

Neutrophils play important roles in host defence against infectious agents but they are also involved in the pathology of various inflammatory conditions. Upon phagocytosis, they produce oxidative and non-oxidative microbicidals for destruction of these agents intracellularly. They also release cytotoxic molecules into the extracellular milieu which can damage the host (Smith, 1994).

Unlike macrophages, the role of neutrophils in the pathogenesis of leprosy has not been well investigated. The role of neutrophils in inflammation and host defence had long been thought to be restricted to phagocytosis and bacterial killing. However, more recent studies have demonstrated that plymorphonuclear cells (PMNs) produce a variety of proteins after being challenged with various stimuli (Oliveira et al., 1999). TNF-α, IL-1β and IL-8 are among the cytokines actively produced by neutrophils *in vitro* (Sakaguchi et al., 2008, Bliss et al., 2010). Knowledge about the role of neutrophils in inflammation is largely based on mice model and translational studies are required to evaluate the clinical importance of these observations in human (Phllipson and Kubes , 2011) since proportion of PMNs circulating in mice is 30% compared to 70% in human (Amulic et al., 2012).

Although neutrophils are generally the first cell type to reach the site of inflammation, the chronic course of leprosy is characterized by the absence of neutrophils *in situ*. An *in vitro* study on the chemotactic responses of neutrophils derived from 52 patients with lepromatous leprosy in Israel and 24 healthy controls has shown that patients with lepromatous leprosy of long-standing had significantly reduced chemotactic responses while those with recently acquired disease showed normal chemotactic responses (Wahba et al., 1980). The significance of neutrophils in leprosy has only been indicated in leprosy reactions particularly in Erythema nodosum Leprosum (ENL) (Goihman-Yahr et al., 1978, Hussain et al., 1995, Oliveira et al., 1999).

2.19.1.4. Complements

The complement system (CS) comprises an assembly of liver-manufactured, soluble and cell-bound proteins made up of more than 30 plasma and cell surface proteins that play a crucial role in host defence against infection. As the name

implies it represents the first line of defence which helps or complements the ability of antibodies and phagocytic cells to clear pathogens from the host (Morgan, 1998). Activation of CS results in opsonisation of pathogens and immune-complexes, recruitment of leukocytes, inflammation, and cell lysis.

Recently complement system have been perceived as a central constituent of innate immunity, defending the host against pathogens, coordinating various events during inflammation, and bridging innate and adaptive immune responses (Ricklin and Lambris, 2007). Complement system not only protect the host against infection but also contribute to the amplification of inflammation if activated in excess or inappropriately controlled (Abe, 2006).

There are three major pathways of complement system: i) the classic pathway (CP), which is usually initiated by antigen-antibody complexes followed by complement C1 activation; ii) the alternative pathway (AP), which is initiated by spontaneous hydrolysis of complement component C3; and iii) the mannose-binding lectin pathway (MBLP), which is activated by recognition of certain microbial polysaccharides. All the three pathways converge at the C3 activation step, leading to the generation of opsonins, inflammatory peptides, and formation of the membrane attack complex (Klaska and Nowak, 2007, Bao et al., 2015).

The activation of complement through either the classical, lectin or alternative pathway converges on the deposition of C3b on the pathogen surface, a key event in complement mediated lysis. The activation of the CS also results in the release of small peptides such as C3a and C5a derived from the cleavage of CS proteins which have important biological properties such as mast cell degranulation and release of vasoactive amines resulting in vasodilatation and also chemotaxis of immune cells. Deposition of C3b on the pathogen/target cell initiates a cascade resulting in the assembly of the membrane-attack complex (MAC), forming pores on the surface of the target cell and its eventual osmotic death (Bao et al., 2015).

Complements are probably the first immune systems experimentally studied in leprosy by Lweis and Aronson (1923). The possible role of complements in the pathogenesis of leprosy was investigated by the increased level of activated C_3 in the sera of lepromatous leprosy patients compared to the sera of tuberculoid leprosy

patients. Petchclai et al. (1973) and Schlesinger and Horwitz (1990), showed that phagocytosis of *M. Leprae* is mediated by complement receptors CR1 and CR3 on human monocytes and macrophages in serum.

Gomes et al. (2008), investigated on the levels of complement components and the total complement haemolytic activity in the sera of 91 patients with different clinical forms of leprosy (36 LL, 33 TT, 22 borderlines) and 31 healthy controls. Reduced complement haemolytic activity and lower level of C4 was observed in the sera of patients with LL as compared to patients with TT and borderlines. Furthermore, when a haemolytic assay was used under conditions favouring activation of the alternate pathway of complement activation, no significant difference was observed among all groups suggesting the importance of either the classical or the lectin pathway in leprosy. In the same study, the highest median level of mannan-binding lectin (MBL) were observed in the sera of patients with LL which had lower haemolytic activity whereas patients with TT with lower MBL levels showed higher haemolytic activity. It has been reported that low level of mannan-binding lectin confer partial protection against some intracellular pathogens, such as *M. leprae* and *M. tuberculosis* (Kilpatrick, 2002).

2.19.1.5. Cytokines and chemokines

Cytokines are low molecular weight soluble proteins which mediate the cross-talk between the different cells of the immune system. They play a central role in the recruitment of the immune cells, the clonal development of the lymphocytes, the innate immune response and the effector response of most immune cells. The complex regulatory network of cytokines often determines the clinical course of infections and the outcome.

In leprosy cytokine research focused mainly on the association of different cytokine profile within the spectrum of the disease specifically with the Th1-Th2 cytokine profiles (Barnes et al., 1992b, Fink et al., 1996, Moubasher et al., 1998a). However, recent studies have shown that a distinct cytokine profile associated to a specific clinical form and reactions has not been attained (Misra et al., I995, Nath et al., 2000).

A serum cytokine profile study in 56 untreated Egyptian leprosy patients has reported that IFN-γ and TNF-α were elevated in patients with TT as compared to patients with LL and these two cytokines were negatively correlated with the bacilli index (BI) in these patients. Patients with LL had a significantly higher serum levels of IL-2R, IL-10, and IL-1β and a significant positive correlations with the BI. Significantly high level of IFN-γ and TNF-α were reported in PB, and IL-1β and IL-10 in MB leprosy among untreated 38 PB and 13 MB Indian leprosy patients suggesting the importance of cytokines in classifying the various forms of leprosy and monitoring chemotherapy (Madan et al., 2011a). TNF-α has shown to be necessary in granuloma formation and mycobacterial elimination through stimulating the migration of immune-cells to the infection site (Mohan et al., 2001, Ehlers, 2005). Hence, the granuloma formation and *M. leprae* bacilli elimination in PB could be attributed to the high secretion of TNF-α in these clinical forms of leprosy.

The predominance of IL4, IL-5 and IL-10 in patients with LL is associated with the immune suppression state of lepromatous leprosy. IL-10 has been demonstrated to supress macrophage mediated destruction of intracellular pathogens (Redpath et al., 2014) and IL-4 has been shown to play a role in the Th2 polarization of $CD4^+$ T-cells (Cote-Sierra et al., 2004).

Chemokines are small (8-10 kilo Daltons, kDa) protein molecules that form a subfamily of the cell signalling molecules or cytokines. They are potent chemo-attractants of various leukocyte subsets and play an important role in promoting migration of effector cells in inflammatory diseases. Their profile in leprosy has not been well investigated unlike in other infectious diseases like tuberculosis and leishmaniasis (Oghumu et al., 2010, Slight and Khader, 2013). Kaplan et al. (1987b) have reported that although IP-10 was not constitutively expressed in LL lesions, administration of PPD or IFN-γ into the lesions resulted in a strong induction of IP-10 expression especially by keratinocytes followed by monocytes, fibroblasts and endothelial cells in 65 lepromatous leprosy patients in India. On the contrary, TT lesions showed intense IP-10 expression by keratinocytes and infiltrating cells suggesting a differential expression of IP-10 across the leprosy spectrum associated with IFN-γ expression (Kaplan et al., 1987a). IP-10 is an essential chemokine in

the migration of monocytes and activated T-lymphocytes and also enhanced T-cell adhesion to endothelial cells (Kawaguchi et al., 2015). Monocyte chemoattractant protein-1 (MCP-1) and IL-8 are two other chemokines studied in leprosy and they were found to be elevated in the sera of patients with LL (Lew et al., 2002, Hasan et al., 2004, Hasan et al., 2006).

2.19.1.6. Phagocytosis and antigen presentation

In previous sections it has been described that *M. Leprae* lives in macrophages, monocytes and Schwann cells. Phagocytosis of *M. leprae* by macrophages and Langerhans cells in the skin is mediated by complement receptors CR1 and CR4 on monocytes and macrophages interacting with complement component C3 fixed to the PGL-1 of *M.leprae* (Schlesinger and Horwitz, 1991a, Schlesinger and Horwitz, 1991c). Langerhans cells are a subset of dendritic cells (DC) that initiate immune responses in the skin. Studies have shown that PGL-1 is involved in the invasion of Schwann cells through the basal lamina by binding to laminin-2 and its receptor α-dystroglycan (Ng et al., 2000).

M. leprae can resist lysosomal digestion by inhibiting phagosome/lysosome fusion inside the macrophage (Sibley et al., 1987, Degang et al., 2014). In addition, lipid components form a waxy coating around the bacteria and contribute to the resistance to desiccation and exposure to osmotic imbalance and extremes of pH (Sibley et al., 1987, Degang et al., 2014). Mechanisms underlying phagosomal arrest in mycobacterial phagocytosis have not been identified. Secretion of low molecular weight metabolite by bacteria, expression of enzyme capable of modifying a host component involved in regulation of endosomal interactions or modifications of the phagosomal membrane by lipids present on the mycobacterial outer surface are suggested as mechanisms of phagosomal arrest (Russell et al., 2002).

M. leprae antigens are presented via MHC class I and II pathways. *M. leprae* antigen presentation through MHC class I to $CD8^+$ T-cells results by lysis of the infected cells by catalytic T- cells ($CD8^+$ T-cells). Presentation via MHC class II to $CD4^+$ T-cells results in secretion of IL-2 and IFN-γ followed by activation of macrophage and an influx of cells into the lesion. Human Schwann cells are able to

process and present *M. leprae* antigens through MHC class II to $CD4^+$ T-cells and are efficiently killed by activated T-cells which may potentially cause destruction of Schwann cells and subsequently distraction of nerves (Spierings et al., 2000).

Mycobacterial antigen presentation is not restricted to MHC pathways. Peptide antigens are presented vial MHC class I and II. Lipid and glycolipid mycobacterial antigens are presented via CD1 molecule. It has been shown that CD1 restricted $CD4^+$ T-cell lines from the skin lesions of leprosy patients found to produce IFN-γ suggesting that $CD4^+$CD1-restricted T-cells in leprosy skin lesions are producing Th1-like cytokine pattern. Interestingly, compared to the MHC molecules, many different T-cell subsets, including $CD4^-$, $CD8^-$, TCRαβ and TCRγδ recognize antigens presented by CD1 in addition to $CD4^+$ and $CD8^+$ T-cells (Young and Moody, 2006).

The antigen delivery mechanism to the CD1 and MHC class 1 pathways are not clearly elucidated and there is little information on how *M. leprae* antigens reach the cytoplasm for presentation by MHC-1. In contrast to MHC-II, which picks and process peptide antigens from within the endocytic environment, MHC-I is traditionally considered the primary mechanism by which cytosolically derived antigen can be processed and presented to T-cells. It is likely that *M. leprae* antigens enter the cytosol through pores in the phagosome although there could be other possible mechanisms. It has been shown that *Mycobacterium tuberculosis* (Mtb) escape the phagosome and reside in the cytosol which was postulated as the primary mechanism by which Mtb antigens enter the MHC-I processing and presentation pathway (Harriff et al., 2012).

2.19.2. The Adaptive Immune System and Leprosy

The adaptive, also known as acquired, immune response requires the specific recognition of foreign antigens which activates the humoral response, resulting in B-cell maturation and antibody production, and the cell-mediated response, focussing mainly on T-cell activation. These divergent arms of the host response to pathogens are not mutually exclusive as T-cell help is required for antibody maturation and isotype switching while B-cells can function as antigen-presenting cells in the induction of specific T-cells.

2.19.2.1. Humoral immunity

B-cells within the lymphoid tissue of the body such as bone marrow, spleen and lymph nodes, are stimulated by antigenic substances to proliferate and transform into plasma cells and the plasma cells in turn produce immunoglobulins (IgA, IgD, IgE, IgM and IgG,). Immunoglobulins are also called antibodies.

The study of the humoral immunity in leprosy has largely been restricted to antibodies. Patients towards lepromatous leprosy pole of the spectrum have higher antibody concentration as compared to with the tuberculoid pole. Elevation of the polyclonal isotypes of these classes of antibody types with the highest concentration in patients with LL forms compared to the other clinical types of the spectrum (Turk and Bryceson., 1971, Touw et al., 1982, Jadhav et al., 2011, Kumar et al., 2014a).

Although the in-situ presence of plasma cells and B-cells have been reported in leprosy by Ayer et al., the role of these cells in the pathology of leprosy lesions is unclear. Both Plasma cells and B-cells have been detected in tuberculoid and lepromatous leprosy lesions (Iyer et al., 2007). It was speculated that these lesional B-cells could influence T-cell responses and /or play a role in maintaining the inflammatory reaction in leprosy partly through the local secretion of antibodies. However, data supporting such hypothesis are lacking. It is generally thought that antibodies against *M. leprae* components do not play a significant role in protection against leprosy. However, antibodies may play a role in the uptake of *M. leprae* by mononuclear phagocytes and hence the pathogenesis of the diseases (Schlesinger and Horwitz, 1990).

Memory B-cells

Memory B-cells are B-cell sub-types that are formed within germinal centres following primary infection and are important in generating an accelerated and more robust antibody-mediated immune response in the case of re-infection also known as a secondary immune response. Recent advances in tracking antigen-experienced memory B cells have shown the existence of different classes of memory B-cells that have considerable functional differences. Currently there are three types of memory B-cells: resting, activated and tissue like memory B cells, (Kurosaki et al., 2015). Activated memory B-cells have been shown to function as

effective APCs to naive T-cells (McAdam et al., 1998). Tissue-like memory B-cells (TLM) expressed patterns of homing and inhibitory receptors similar to those described for antigen-specific T-cell exhaustion. These tissue like memory B-cells proliferate poorly in response to B-cell stimuli, which is consistent with high-level expression of multiple inhibitory receptors. Higher percentage of TLM has been reported in immunosuppressive diseases such as HIV (Moir et al., 2008, Amu et al., 2014). During reviewing the literature on B-cell studies, we couldn't find any literature dealing with memory B-cells and leprosy. Hence, memory B cells are potential future areas to elucidate the role of B-cells in leprosy and leprosy reactions.

2.19.2.2. Cell-mediated immunity

T-cells are produced in bone marrow and complete their development in the thymus. Once they completed their development, they enter the blood stream. When reached a peripheral lymphoid organ, they leave the blood to migrate through the lymphoid tissue, returning via the lymphatics to the bloodstream to recirculate between blood and peripheral lymphoid tissues. The stimulation and clonal expansion of a specific T-cell response occurs mainly in the peripheral lymphoid organs, where circulating naïve T-cells from the blood come in contact with antigens presented by antigen presenting cells such as macrophages, dendritic cells or B-cells depending on the nature of the antigen to be presented (Murphy et al., 2012).

On antigen recognition, naive T-cells differentiate into either CD4 or CD8 effector T-cells that are specialized for different activities. CD8 T-cells recognize pathogen peptide presented by major histocompatibility class one (MHC I) molecules and naive CD8 T-cells differentiate into cytotoxic effector T-cells that recognize and kill infected cells (Wang et al., 2001) . CD4 T-cells have a wide range of effector activities. After recognition of pathogen peptides presented by MHC II molecules, naive CD4 T-cells differentiate into district pathways that generate effector subsets with different immunological functions. The main $CD4^+$ effector subsets currently distinguished are Th1, Th2, Th17, follicular T helper cells (Tfh) and regulatory T-cells (Tregs) (Figure 2.11). Th1, Th2, Th17 and Tfh activate their target cells while

Tregs convey an inhibitory activity that limits the extent of immune activation (Kim and Cantor, 2014) (Figure 2.10).

Effector $CD8^+$ T-cells, employ antimicrobial effects either by secretion of cytokines such as IFN-γ and TNF-α activating T-cells or by direct mechanisms such as the perforin/granzyme pathway-mediated lysis of pathogen-infected cells. However, a subset of $CD8^+$ T-cells with regulatory or suppressor activity has been reported by several authors (Xystrakis et al., 2004, Wang and Alexander, 2009, Menoret et al., 2011, Boer et al., 2014).

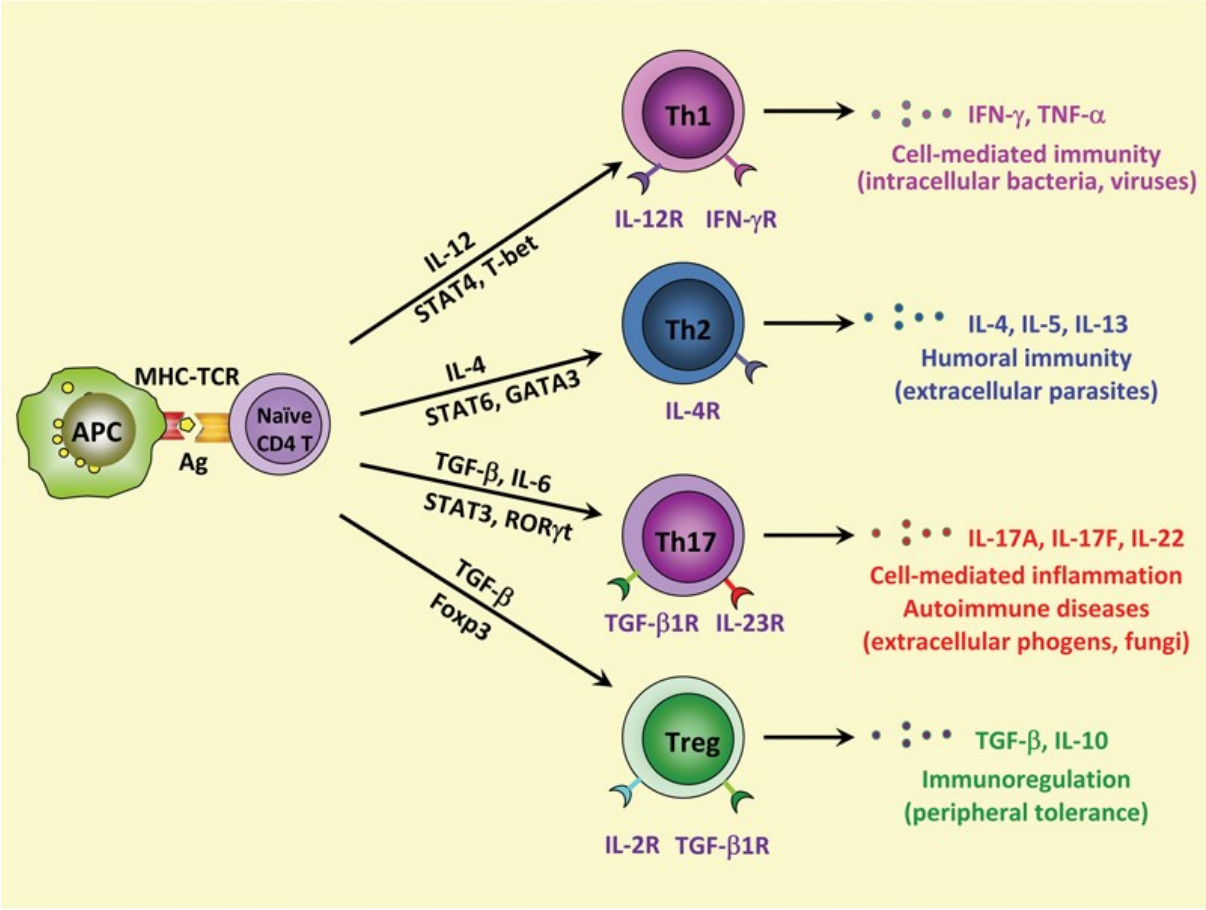


Figure 2.10. Schematic model for helper cell differentiation from naïve CD4 T-cells (Leung et al., 2010)

The classification of $CD4^+$T- helper cells into Th1 and Th2 was initially based on the type of cytokines produced by these subsets in mice models (Mosmann and Coffman, 1989). Later on, they were also described in human cells with similar cytokine profiles to the pervious findings in mice model (Romagnani, 1991). Human Th1 cells produces IL-2, a T-cell growth factor; IFN-γ, a main macrophage activating factor; and TNF-α which initiates granuloma formation (Romagnani, 1991, Leung et al., 2010). The Th2 subset of $CD4^+$ T-cells secretes IL-4, which is an autocrine growth factor, IL-5 and IL-6 which give signal for the differentiation and proliferation of B-cells (Romagnani, 2000).

Little is known about the activation and differentiation process of Th1/Th2. It is thought to be influenced by the type of antigen presenting cell and by the cytokine milieu at activation. It has been shown that antigen presentation by macrophage produces a Th1 response, whereas antigen presentation by B-cells induces a Th2 response in mice model with ovalbumin (Gajewski et al., 1991). Although the balance between Th1 and Th2 is crucial for the successes of the immune response in terms of the specificity and magnitude, it has been remained mysterious how the Th1/Th2-response profile is matched to district pathogens and to particular affected tissues (Pulendran, 2004, Magombedze et al., 2014).

It has been described that the cytokines secreted by Th1 and Th2 show cross-regulatory property for the differentiation and effector functions of the mutual phenotype. For example, IFN-γ selectively inhibits Th2 cells proliferation, while IL-4 and IL-10 inhibits cytokine secretion by Th1 cells (Fishman and Perelson, 1999, Magombedze et al., 2014). Generally, the induction of Th1 during intracellular infection leads to protection while induction of Th2 associated to diseases progression.

2.19.2.2.1. Cell- mediated Immunity and leprosy

Leprosy has been studied as a model system to study the Th1/Th2 hypothesis. Several independent studies (Ochoa et al., 1996, Moubasher et al., 1998a, Volc-Platzer et al., 1988, Madan et al., 2011c) have shown that the infiltrating T-cells in TT leprosy produced IL-2 and IFN-γ within the lesions. These T-cells were not found in lepromatous lesions. Analysis of gene expressions of IL-2 and IFN-γ also demonstrated a difference between TT and LL lesions. The mRNA expression for

IL-2 and IFN-γ was remarkably higher in TT lesions, whereas, IL-4, IL-5 and IL-10 mRNAs expression were significantly higher within the lesions of LL compared with TT lesions (Yamamura et al., 1991, 1992). These finding suggest that the cytokines produced by Th1 subset in TT lesions correlated with healing tuberculoid lesions, whereas, a Th2 profile correlated with progressive in the lepromatous leprosy.

On the other hand, many investigators reported that majority of patients showed a co-expression of IFN-γ and IL-4 irrespective of the clinical forms of the spectrum (Misra et al., I995, Nath et al., 2000). However, it must be noted that when bulk PBMCs and mononuclear cells are stimulated with various *M. leprae* antigens, it may not necessarily reflect the situation of cells at the clonal level or within the lesion. Although there is some evidence for association of polarized Th1 and Th2 responses in leprosy spectrum, in T-cell populations such distinction may be less noticeable *in vivo* due to the complexity of the induced immune response (Iyer et al., 2007).

2.19.2.2.2. Helper T-cells and cytotoxic T-cells in leprosy

Immunohistochemical studies have shown that $CD4^+$ T-cells are the predominant subset in patients with TT whereas the majority of the T-lymphocytes population in patients with LL forms are of the $CD8^+$ subtype (Modlin et al., 1983, 1986) . A study by Van Voorhis et al. (1982) showed that the T-cell infiltrate in LL lesions consisted almost exclusively of OKT8/Leu-2a cells in contrast to the predominance of OKT4/Leu-3a helper T-cells in TT lesions. Similar observation were also reported in subsequent studies (Narayanan et al., 1983, 1984).

Moreover, it has been found that $CD4^+$ T-cell clones from TT individuals proliferated in response to *M. leprae* antigens whereas $CD8^+$ T-cell clones from lepromatous individuals did not proliferate but suppressed the proliferation of $CD4^+$ T-cells (Damle et al., 1984, Modlin et al., 1986, Salgame et al., 1991). However, one earlier study by Molloy et al. (1990) reported that the suppression of T-cell proliferation by *Mycobacterium leprae* is monocyte-dependent+ but not $CD8^+$ T-cells dependent.

2.19.2.2.3. Markers for identification of regulatory T-cells

Regulatory T-cells (Tregs) formerly called suppressor T-cells are subpopulations which modulate the immune system and maintain tolerance to self-antigens. Tregs are either produced in the thymus as a functionally mature subpopulation of T-cells and or induced from naive T-cells in the periphery (Oliveira et al., 1999, Shevach, 2009). Tregs play an important role in the mechanism of host's failure to control pathogen dissemination in severe forms of different chronic granulomatous diseases. While Tregs require antigen specific activation to carry out their effector function, their suppressive effects are mediated in a non-specific manner inhibiting both innate and adaptive immune responses (Martin et al., 2003, Tanriver et al., 2009, Lehtimäki and Lahesmaa, 2013).

Despite these reports, the presence and activity of Tregs and their subsets has not been conclusively demonstrated due to the lack of an absolute marker for these Tregs. Different combinations of cellular markers have been used by different researchers but none of them found to be an absolute marker. The difficulty of finding the best marker for Tregs may be explained by the complex nature of the immune regulation and the plasticity of immune cells which are perhaps controlled by the environment in which they are operating.

Initially it was suggested that $CD4^+CD25^+$ T-cells as a good markers for Tregs but it was found that upon activation, all T-cells express CD25 (the α-chain of the IL-2 receptor) (Sakaguchi et al., 1995, Yamazaki et al., 2003). The concept of using $CD4^+CD25^+$ as a marker for regulatory T-cells was derived from the results obtained from mice model. In mice, $CD4^+$ Tregs are a homogenous population, in which all $CD4^+CD25^+$ T-cells are regulatory T-cells. In humans, the Tregs are heterogeneous population, in which not all $CD25^+$ T-cells are Tregs. This was first observed by a detailed analysis of human $CD4^+CD25^+$ populations by Baecher-Allan et al. (2005). Consequently it was demonstrated that CD25 is highly upregulated on T-cells upon stimulation (Corthay, 2009). The concept of $CD4^+CD25^{high}$ as a marker for Tregs was introduced following the observation of the *in vitro* suppression of $CD4^+CD25^{high}$ in human cells similar to that described in murine cells while the cells expressing low-to-intermediate levels of CD25 did not (Baecher-Allan et al., 2005). However, a consensus on the thresholds of CD25

expression needed to delineate Treg cells within the $CD25^{high}$ population is difficult to attain, and variations in FoxP3 expression within the $CD25^{high}$ population have been observed even in healthy individuals (Miyara et al., 2009). Hence, the CD25 high and low is highly subjective and it leads to subject bias.

It was also suggested that the immune-suppressive function of $CD4^+CD25^+$ T-cells was dependent on signalling via the down regulation of T-cell activation by cytotoxic lymphocyte- associated antigen-4 (CTLA-4) (Read et al., 2000). These findings raise the possibility that Tregs function contributes to the immune suppression characteristic of CTLA-4 signalling. However, CTLA-4 is also induced on $CD4^+$ T-cells upon activation and hence increases the false positivity of Tregs measurement (Dhuban and Piccirillo, 2014) .

The Ikaros family transcription factor Helios, was suggested to be exclusively expressed by Treg cells (Thornton et al., 2010), although later studies challenged this notion by reporting Helios up-regulation in *in vitro*-induced Treg cells (iTreg), which are thought to be generated from conventional T-cell precursors in the presence of TGF-β (Akimova et al., 2011).

It was shown that low expression levels of CD127, in conjunction with CD25 expression, provides highly enriched $FoxP3^+$ population that encompasses the majority of $FoxP3^+$ T-cells (Liu et al., 2006). But it has been reported that the stimulation of human T-cells may down- regulate CD127 (Miyara et al., 2009, Yu et al., 2012). FoxP3 remains as a good candidate marker of Tregs although the heterogeneity of FoxP3 is also coming to the picture. The concept of natural regulatory T-cells (nTregs) and inducible regulatory T-cells (iTregs) further complicated the search for reliable biomarker for Tregs. $FoxP3^+Helios^-$ and $FoxP3^+Helios^+$ are suggested as a marker for nTregs and iTregs respectively (Elkord et al., 2015). It has also been shown that Tregs are not confined to only $CD4^+$ T-cells. $CD8^+$ regulatory T- cells have had a long history in immunology as suppressor T-cells. Like $CD4^+$ Tregs, the concept of natural and induced $CD8^+$ Tregs has also recently been introduced (Ndure and Flanagan, 2014).

2.19.2.2.4. Regulatory T-cells in leprosy

Although, studies on the association of regulatory T-cell phenotypes with leprosy are lacking, an *M. leprae* specific suppression of effector response has been described before the characterisation of Tregs by several researchers (Sakaguchi, 2003). The first report was made by Mehra et al. (1979) when they noticed the suppression of proliferative responses to concanavalin A (an immunogenic lectin carbohydrate binding protein) in the presence of lepromin in LL and BL patients.

Quantification of Tregs in the PBMCs stimulated *in vitro* by *M. leprae* antigenic preparations and phytohemagglutinin (PHA) by flow cytometry as well as in the skin lesions by immunohistochemistry showed that *M. leprae* antigens induced low lymphoproliferative response (low mean cell counts per minute) but high number of Tregs in lepromatous patients than in tuberculoid patients (Brandt et al., 2000). A cell subset analysis and confocal microscopy of skin biopsies in Ethiopian leprosy patients showed increased frequencies of Tregs in the blood as well as in the lesions of LL patients compared to TT and borderline leprosy lesions (Bobosha et al., 2014b). Similar result has been reported in Indian patients by Saini et al. (2014b) and in Brazilian patients by Parente et al. (2015).

2.19.2.2.5. Th-17 cells in leprosy

IL-17-producing T-cells have recently been classified as a new effector T-cell subset, termed Th17, which is distinct from Th1, Th2 and Treg sub-sets. The differentiation of naïve CD4+ T-cells to IL-17 producing T-cells in the presence of IL-6 and TGF-β by disfavouring Th1 and Th2 was first described by Veldhoen et al. (2006). Th-17 cells produce IL-17, IL- 22 and IL-21 effector cytokines. IL-17 is a member of a family of cytokines with at least 6 members in the human genome, including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. IL-17 is considered as a pro-inflammatory cytokine because it increases IL-6, IL-8, nitric oxide, TNF-α and IL- 1β production by various cell types. It has been reported that IL-17 plays key role for activation and recruitment of neutrophils to the site of infection in inflammatory diseases (Miyamoto et al., 2003). The involvement of IL-17 as pro-inflammatory cytokine in human inflammatory diseases such as rheumatoid arthritis, psoriasis, crohn's diseases, systemic lupus erythematosus, inflammatory bowel diseases and multiple sclerosis has been reviewed by Miossec

(2009). It has been described that Th17 and Tregs have reciprocal functions. Th17 induce inflammation in autoimmune diseases while Tregs inhibit autoimmune tissue damage (Bettelli et al., 2006). Recently it was reported that Th-17 cells transdifferentiate into regulatory T-cells during resolution of inflammation by a change in their signature transcriptional profile and the acquisition of potent regulatory capacity (Gagliani et al., 2015).

A decreased mRNA expression of IL-17A in the skin lesions of patients with leprosy compared to the endemic healthy controls were reported by da Motta-Passos et al. (2012) with undetectable IL-17A in the serum of both patients and controls. In these leprosy patients, lepromatous leprosy patients had low level of IL-17A mRNA expression which had an inverse linear correlation with bacilli index (BI). These authors suggested that the low mRNA expression for IL-17A in patients may be a constitutive genetic feature of leprosy patients or a circumstantial event induced by the local presence of the pathogen, as an escape mechanism. On the contrary, another study reported that the level of IL-17A in the serum of untreated leprosy patients (TT, BT and LL) was successfully measured by ELISA and the lowest amount of IL-17A was measured in LL cases (Abdallah et al., 2013). Similarly, IL-17 isoforms (IL17A-F) showed significantly higher expression and release in supernatants of antigen stimulated PBMCs cultures and dermal lesions of healthy contacts and tuberculoid leprosy as compared to lepromatous leprosy (Saini et al., 2013).

2.19.2.2.6. Memory T- cells

Adaptive immunity is said to have memory because the immune system learns. Some effector cells in both B and T cells differentiate into memory cells following infection. However, memory T-cells are extensively studied unlike memory B-cells. Following infection, some of the activated T-cells become memory cells that exist in a state of readiness and have the ability to rapidly expand and fight off recurrence of the same disease (Figure 2.11). In a way, these cells learn from their experience of fighting a particular infection and so can use the most effective strategy to manage the same infection later. This ability to remember and learn is exploited during the process of vaccination.

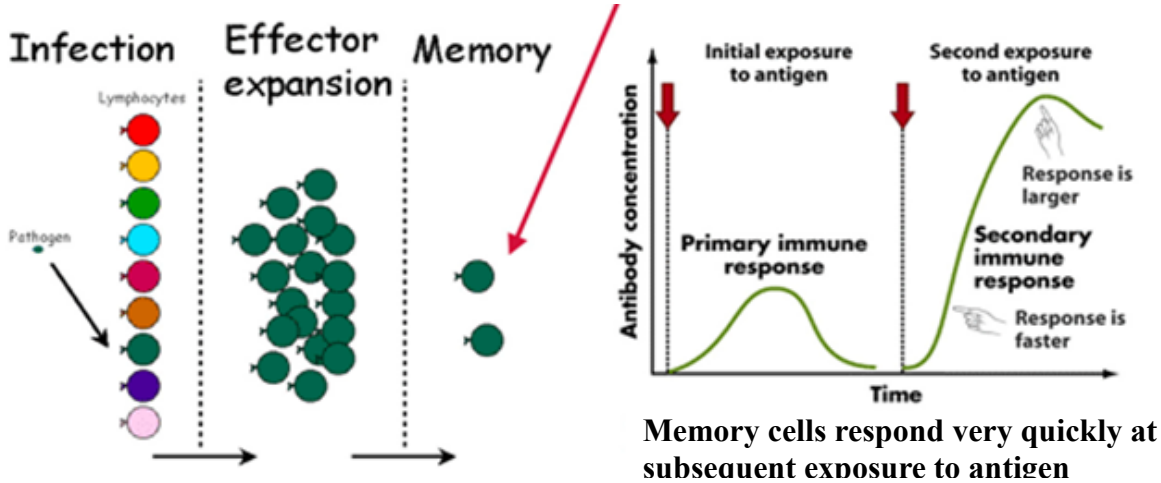


Figure 2.11. The generation of memory cells (Farber et al., 2014).

Memory T-cells have been characterized by their phenotypic and functional profiles into T-cell subsets, typically central memory (T_{CM}) and effector memory (T_{EM}) T-cells (Table 2.5). T_{CM} and T_{EM} cells phenotypically differ by the presence of lymph node homing receptors CD2L (L-selectin) and C-C chemokine receptpr-7 (CCR7) on their surface. CD62-L and CCR7 are present on the surface of T_{CM} but not on the T_{EM} cells (Table 2.7). Naïve (N_{TC}) and T_{CM} express CD62L and CCR7 for migration to secondary lymphoid organ, and in the absence of these molecules T_{EM} and effector T cells (T_{EC}) accumulate in the peripheral tissues. Central memory T-cells produce IL-2 whereas, effector memory T-cells have increased secretion of effector cytokines such as IFN-γ and IL-4. T_{CM} cells are relatively long-lived memory cells, which are able to differentiate into shorter-lived T_{EM} cells upon antigen stimulation. In turn, T_{EM} cells differentiate into T_{EC} cells. T_{EC} cells represent terminally differentiated T_{EM} cells, and death is the outcome of increased antigen exposure and proliferation of T_{EC} cells (Farber et al., 2014, Flynn and Gorry, 2014).

Stem memory T-cells (T_{SCM}) have recently been described as sets of memory T-cells in mice and humans consisting 2-4% of $CD4^+$ and $CD8^+$ T-cells population in the periphery. They thought to represent the earliest and long-lasting developmental stage of memory T-cells, displaying stem cell-like properties, and exhibiting a gene profile between naïve and central memory T-cells (Flynn and Gorry, 2014).

Table 2.5. Phenotypic characteristics of T-cell subsets

Naïve	TSCM	T_{CM}	T_{EM}	EC
$CD45RA^{+}$	$CD45RA^{+}$	$CD45RA^{-}$	$CD45RA^{-}$	$CD45RA^{+}$
CD45RO-	$CD45RO^{+}$	$CD45RO^{+}$	CD45RO+	CD45RO-
$CCR7^{+}$	$CCR7^{+}$	$CCR7^{+}$	$CCR7^{-}$	$CCR7^{-}$
$CD62L^{+}$	$CD62L^{+}$	$CD62L^{+}$	$CD62L^{-}$	$CD62L^{-}$
$CD27^{+}$	$CD27^{+}$	$CD27^{+}$	$CD27^{-}$	$CD27^{-}$
$CD28^{+}$	$CD28^{+}$	$CD28^{+}$	$CD28^{+/-}$	$CD28^{-}$
$CD95^{-}$	$CD95^{+}$	$CD95^{+}$	$CD95^{+}$	$CD95^{+}$
$CD122^{-}$	$CD122^{+}$	$CD122^{+}$	$CD122^{+}$	$CD122^{+}$

T_{CM}, Central memory; T_{EM}, effector memory; EC= effector T-cells ;TSCM, stem memory T-cell (Flynn and Gorry, 2014)

Memory T-cells in leprosy: Few studies have intended to identify the memory T- cell subsets in leprosy. One earlier study has shown that in fresh and unstimulated blood leukocytes from leprosy patients, memory T-cells predominated in the PB form of the disease and correlated with IFN-γ production but such result was not observed in MB patients (Mitra et al., 1999b). However, the study did not use an experimental design that allowed discriminating identification of memory T-cell subsets.

The correlation between central memory T-cell expression and pro-inflammatory cytokine production with clinical presentation of multibacillary leprosy relapse case has been investigated by Esquenazi et al. (2015). Increased frequency of central memory T-cells in relapsed patients was strongly correlated with the bacillary index and the number of skin lesions was reported by these authors. The study did not give attention to the memory subsets in leprosy spectrum rather they focused on relapse cases. Memory T-cells have not been characterized across leprosy spectrum as well as in leprosy reactions. In the present study, for the first time we described memory-T cell subsets in lepromatous leprosy and ENL reactions. We also characterized memory T-cell subsets in other forms of leprosy (data not presented in this thesis).

CHAPTER 3: LEPROSY REACTIONS

3.1. Introduction

Leprosy reactions are immune-mediated incidents of acute or sub-acute inflammation and are the main complication of the disease. A significant proportion of leprosy patients, especially in the borderline region of the spectrum, develop leprosy reactions. Leprosy reactions can occur before MDT, during or after successful completion of MDT. There are two types of leprosy reactions:(i) Reversal Reactions (RR), also designated as type 1 leprosy reaction, which occurs mainly in patients with borderline leprosy and (ii) Erythema Nodosum Leprosum (ENL) or type 2 leprosy reaction, which predominantly occurs in patients on the lepromatous side of the spectrum reviewed by Lockwood et al. (1993).

3.2. Type 1 (Reversal) Reactions

Type 1 reactions (T1Rs) manifest clinically with erythema and oedema of skin lesions and tender peripheral nerves with loss of nerve function. They are characterized clinically by acute inflammation of skin and/or nerves leading to nerve impairment if not treated rapidly (Walker and Lockwood, 2006). The characteristic signs of T1Rs are an erythematous swelling of existing lesions, appearance of new lesions and the onset or worsening of neuritis (Naafs, 2000). Histopathologically, T1R is characterized by a shift towards the tuberculoid end of the leprosy spectrum with increased infiltrate of lymphocytes, epithelioid cells, giant cells, oedema and a decrease in bacterial load.

3.1.1. Epidemiology

Type 1 reactions occur in up to 30% of patients with borderline forms of leprosy (Walker and Lockwood, 2006). In a study conducted in Brazil, among 122 leprosy patients investigated 44 (36.1%) had type one reactions (Motta et al., 2012b). In a retrospective study conducted in India, from 494 leprosy patients a clinical evidence of T1R was found in 44 (10.9%) (Lockwood et al., 1993). The ALERT MDT field evaluation study reported that among 594 leprosy patients 16.5% of them had developed T1Rs (Saunderson et al., 2000).

In Nepal, 31% (204/658) leprosy patients with borderline spectrum had a T1Rs during the MDT treatment (Roche et al., 1991b). Similarly, about 36% (50/305) of a cohort of MB patients in Malawi had developed T1Rs during a three year follow-up period (Ponnighaus and Boerrigter, 1995). In INFIR Indian cohort study about 20% (60/303) had T1Rs at recruitment and up to 39% (74/188) had experienced T1R or nerve function impairment (NFI) during the two year follow-up period (Van Brakel et al., 2005b).

3.1.2. Risk factor

The risk of a patient developing T1Rs is higher in the first six months of MDT treatment (Lockwood and Sinha, 1999). A retrospective study from China has indicated that among 159 cases with T1Rs, 66 (41·5%) had occurred during the first 6 months of MDT treatment and only 15 (9·4%) had occurred after 12 months of MDT treatment (Shen et al., 2005). On the other hand, Ethiopian (Saunderson et al., 2000) and Indian (Kumar et al., 2004) cohort studies have shown that patients continue to develop T1Rs and neuropathy in the third year after diagnosis and beyond, regardless of MDT completion.

Patients with borderline leprosy are at higher risk of developing T1R (Ranque et al., 2007). Individuals who have WHO disability grades 1 and 2 at diagnosis are significantly more likely to have severe T1Rs (Schreuder, 1998). Borderline patients with positive slit-skin-smear (SSS) test, detectable *M. leprae* DNA by PCR and positive for anti-PGL-1 antibodies test have an increased risk of T1R (Roche et al., 1991a).

In an Ethiopian cohort study of 431 patients with leprosy, a single nucleotide polymorphism (SNP) in TLR2 (597C→597T) was found to be associated with protection against T1Rs, whereas a 280-bp microsatellite marker was associated with an increased risk of T1Rs (Bochud et al., 2008). In another cohort study of 441 Ethiopian patients with leprosy, TLR4 SNP (1530G → 1530T) was more frequent in individuals with T1R suggesting that TLR4 SNP of G to T as a risk factor for T1R and two single nucleotide polymorphisms in TLR4 (896G →A and 1196C→T were associated with protective effect against T1R (Bochud et al., 2009). An *in vivo* study in Nepalese patients with leprosy has shown an increased TLR2 and TLR4 gene expression in untreated patients with T1Rs suggesting the

involvement of TLR2 and TLR4 in the pathogenesis of T1Rs (Walker et al., 2012). Other host genetic factors such as: HLA-DR B1, nucleotide-binding oligomerisation domain containing 2 (NOD2), vitamin D receptor and natural resistance-associated macrophage protein-1 have been indicated to be associated with T1Rs (Fava et al., 2012).

3.2.3. Diagnosis of Type 1 (reversal) reactions

The diagnosis of T1R is usually clinical but sometimes histological finding is used to supplement the clinical diagnosis. The important diagnostic features used by pathologist in diagnosing T1R include intra-granuloma oedema, giant cell size, giant cell numbers, dermal oedema and HLA-DR expression (Walker and Lockwood, 2006, Lockwood et al., 2008).

In a prospective study based in India, skin biopsies were collected from 99 patients with clinically diagnosed T1R and 52 non reactional controls, pathologists diagnosed T1R only 50% of clinically diagnosed reactions (Lockwood 2007). These authors concluded that T1R is under diagnosed by histology compared to clinical diagnosis. On the other hand, in INFIR cohort study in India, from a total of 265 newly diagnosed leprosy patients, T1R was diagnosed histologically in 42.4% of patients but only in 20.8% clinically (Lockwood et al., 2012).

Explanation for the discrepancy between the two studies is not straight forward. However, it clearly indicates the existing challenges for accurate diagnosis of reactions. Clinical signs heterogeneity within the reaction, lack of standard criteria for histological diagnosis, experience of pathologists and clinicians in leprosy reactions diagnosis could be one of the reasons for the observed difference in these studies. However, these reasons need to be confirmed by further study.

3.2.4. Treatment of Type 1 reactions

The treatment of T1Rs is aimed at resolving the skin lesions and minimizing the risk of permanent nerve damage particularly to the peripheral nerve trunks. According to WHO recommendation, severe forms of T1Rs should be treated with a course of steroids usually for 3-6 months. The usual course begins with 40-60mg daily and then gradually reduced weekly or fortnightly and eventually stopped.

Most reversal reactions and neuritis are treated successfully with a standard 12-week course corticosteroid treatment (World Health Organization, 2006).

3.3. ENL (Type II) Reactions

ENL (Type-2) reaction is an immune-mediated inflammatory complication, occurring in about 50% of LL and 10% BL patients (Van Veen et al., 2009). Murata in 1912 gave the first clinical and histological description of the reactional lesions in leprosy which he referred as erythema nodosum leprosum (ENL) (Penna et al., 2008). However, since the term ENL was descriptive of only the nodular lesion in the skin, Jopling in 1959 proposed the name type II reaction because of the numerous other systemic symptoms associated with the condition (Bwire and Kawuma, 1993). However, ENL is the commonly used terminology in leprosy literature and so, we adhere to this in this thesis.

3.1.1. Epidemiology

ENL reactions can occur before, during or after treatment (Shen et al., 2005). Accurate data on global and regional prevalence of ENL reaction is lacking (Voorend and Post, 2013). In Six prospective and five retrospective field-based studies the cumulative frequency of ENL varied between 0.2% in an Indian study (Rao et al., 1994) and 4.6% in a Chinese study (Shen et al., 2009a). Three prospective studies from ALERT, leprosy control services in Ethiopia reported a cumulative frequency of ENL at 2.5% among MB cases after an average follow-up of 2 and a half years, but 5% after 10-years follow-up (Becx-Bleumink and Berhe, 1992, de Rijk et al., 1994, Saunderson et al., 2000b). In INFIR cohort study of 303 newly diagnosed MB leprosy patients in India, it was found that 17% LL and 7% BL had ENL (Lockwood et al., 2012). These authors reported that ENL diagnosis was made at baseline and highlighted that a higher rate of ENL reactions would be expected if cohort patients were followed-up after treatment.

The frequency of ENL reaction according to the Ridley- Jopling classification has been reported in many studies (Table 3.1 and 3.2). In field studies, higher frequency of ENL is reported in LL patents (15.4%, range: 11.1 to 26%) compared to BL patients (4.1%, range: 2.7 to 5.1%) (Table 3.1). In hospital based studies higher proportions were reported, in Brazil up to 56.4% and in India from 24.2% to 50.9

% (Shen et al., 2009a). High rate ENL frequency (91%) among LL patients has been reported from Brazil showing that ENL is more common in Brazilian LL patients (Nery et al., 1998). In a retrospective study of 481 BL and LL patients in Hyderabad, ENL occurred in 49.4% of LL and 9.0% of BL leprosy cases (Pocaterra et al., 2006).

Some authors suggest that since the introduction of MDT, the frequency and severity of ENL may have been decreased by the anti-inflammatory action of the clofazimine component of MDT (Kahawita and Lockwood, 2008). A prospective cohort study in Philippines reported that among 296 patients treated with MDT for 12 months and 293 patients for 24 months, ENL was not significantly more common, but it was longer-lasting and more severe in patients receiving only 12 months of MDT, as compared with those receiving 24 months treatment (Balagon et al., 2011). This would be compatible with a protective effect of clofazimine.

Table 3.1. Incidence of ENL in Field based studies

country	Follow up period (years)	Number of leprosy of cases	Definition of cases	ENL n(%)	Authors
Prospective observational cohort studies					
Bangladesh	5	337	MB	8(2.2)	(Richardus et al., 2004)
Thailand	>2	133	BL+LL	16(12.0)	(Schreuder, 1998)
Ethiopia	3.5	375	BL+LL	19(5.0)	(Becx-Bleumink and Berhe, 1992)
Ethiopia	2.5-4	158	MB	4(2.5)	(de Rijk et al., 1994)
Ethiopia	10	300	MB	16(5.3)	(Saunderson et al., 2000a)
India	1-7	106	MB	4(3.8)	(Rao et al., 1994)
Retrospective observational cohort studies					
Bangladesh	n/a	471	MB	10(2.1)	(Richardus et al., 1996, Desikan et al., 2007)
India	7	1067	BL+LL		(Desikan et al., 2007)
Cross-sectional studies					
China	n/a	726	MB	9(1.2)	(Shen et al., 2009a)
Indonesia	n/a	586	MB	13(2.2)	(Bernink and Voskens, 1997)
Total (average)		4,279		194(4.5)	

n/a: data not available

Source: Table 3.1.and 3.2 adapted from (Voorend and Post, 2013).

Table 3.2. Incidence of ENL cases in hospital populations

country	follow-up period years	leprosy cases (no)	definition	ENL N (%)	Authors
Retrospective observational cohort studies					
India	>2-10	578	BB+BL+LL	164 (28.4)	(Kumar et al., 2003)
India	>1	481	BL+LL	117(24.4)	(Pocaterra et al., 2006)
Philippines	4	296	MB	36 (12.2)	(Balagon et al., 2011)
Nepal	2	175	BL+LL	10(5.7)	(Van Brakel et al., 1994)
Brazil	2	169	BB+BL+LL	43(25.4)	(Nery JAC et al., 199)
Brazil	2	162	BB+BL+LL	51 (31)	(Nery et al., 1998)
India	2	990	BB+BL+LL	121(12.2)	(Salodkar and Kalla, 1995)
India	3-13	1494	MB	337(22.5)	(Kumar et al., 2004)
Brazil	unknown	218	MB	28 (13)	(Katoch et al., 2008)
Prospective observational cohort studies					
India	2	303	MB	6(2)	(Van Brakel et al., 2005b)
India	<8	980	MB	2(0.2)	(Vijayakumaran et al., 1995)
Thailand	3	119	BL+LL	44(37.0)	(Scollard et al., 1994)
India	6	100	MB	6(6)	(Katoch et al., 2008)
Philippines	3	169	MB	10 (7.0)	(Balagon et al., 2010)
Cross-sectional studies					
Uganda	n/a	2743	MB	18 (0.7)	(Bwire and Kawuma, 1993)
India	"	1141	MB	187 (16.4)	(Arora et al., 2008)
Nepal	"	563	BL+LL	107(`19)	(Manandhar et al., 1999)
Brazil	"	664	MB	192 (28.9)	(Penna et al., 2008)
Netherlands	"	231	BB+BL+LL	17(7.4)	(Post et al., 1994)
Morocco	"	229	MB	76(33)	(Hali F et al., 2009)
India	"	187	BB+BL+LL	25(13.3)	(Sharma et al., 2004)
Yemen	"	123	BB+BL+LL	33(26.8)	(Mekhlafi and al-Qubati, 1996)
Brazil	"	120	MB	13(10.8)	(Rodrigues et al., 2000)
Controlled trails					
Thailand, Philippines, Korea	5	358	BL+LL	36 (10)	(Cellona et al., 1990)
India	8	304	BB+BL+LL	30 (10)	(Sharma et al., 2004)
Zaire	3	280	MB	34(12)	(Groenen et al., 1986)
Brazil	2	140	MB	48 (34.2)	(Gallo et al., 1996)
	Total	13317		1791(13.4)	

3.3.2. Risk factors

Studies have shown that a BI > 4 significantly increases the risk of developing ENL and the degree of skin infiltration correlates positively with risk of ENL (Manandhar et al., 1999). For individuals with LL and BL with BI ≥ 6, the odds of developing ENL was 8.4 and 5.2 respectively (Pocaterra et al., 2006). Similarly, an Ethiopian study reported that the ENL frequency was 9.6 times higher among LL patients compared to BL and BB (Becx-Bleumink and Berhe, 1992). In a retrospective study of 563 Nepali patients, the greater infiltration of the skin and BI > 4 reported as a high risk factor for developing ENL reactions (Manandhar et al., 1999).

Pregnancy and lactation suggested as significant triggering factors for severe and recurrent ENL(Lockwood and Sinha, 1999). The frequency of ENL among Ethiopian pregnant women increased by 59% among LL and 22% among BL (Duncan and Pearson, 1984). An Indian study reported that among 32 women with ENL, 62% were either pregnant or lactating (Arora et al., 2008). Intrecurrent infections, vaccination, stress, genetics and age (puberty) have also been implicated as a risk factor for ENL (Kahawita and Lockwood, 2008, Motta et al., 2012a).

The host genetic factors as a risk factors for ENL have not been addressed. Some studies have shown the associations of some genes with ENL such as variants in vitamin D receptor (VDR) genes, IL-6, Nucleotide-Binding Oligomerization Domain Containing-2 (NOD2) and complement C4B. In a cohort of 409 patients with leprosy in Brazil, evidence for association between ENL reaction and *IL-6* single-nucleotide polymorphisms rs2069832, rs2069845, rs2069845 and rs1800795 which correlates with increased IL-6 plasma production has been reported. Such association was not found inT1R (Sousa et al., 2012). Low vitamin D receptor expression level has been reported to be correlated with ENL reaction in Indian patients (Mandal et al., 2015). A cross-sectional study in Nepalese patients, examined polymorphisms in the NOD2 gene region among 124 patients with ENL reaction and 428 non-reactional patients with LL/ BL has found that *NOD2* genetic variants are associated with ENL reaction (Berrington et al., 2010). One study which included 72 Brazilian patients with leprosy (43 ENL and 36 non-reactional LL) has reported that *C4B* deficiency (*C4B*Q0*) was significantly higher

among patients with ENL compared to LL suggesting that C4B deficiency may affect both the phagocytosis during the reactivation of the inflammatory response in ENL reaction and the stimulation of cellular immune responses, possibly through IP-10-related pathways (de Messias et al., 1993). A Brazilian study consisting of 201 leprosy cases has shown that the SNP 274T→ C of *NRAMP1* as a risk factor for T1R while being protective for ENL (Teixeira et al., 2010) .

3.3.3. Clinical features of ENL reactions

ENL usually affects multiple organs and causes systemic illness (Walker and Lockwood, 2006). The onset of ENL is acute, but it may pass into a chronic phase and can be recurrent. ENL produces fever and in the skin painful and tender red papules or nodules occur in crops often affecting the face and extensor surfaces of the limbs. The lesions may be superficial or deep causing a panniculitis. ENL reactions may also produce uveitis, neuritis, arthritis, dactylitis, lymphadenitis and orchitis. The recurrent inflammation of eyes can lead to blindness and the testes to sterility (Walker and Lockwood, 2006).

Reoccurrence (episode) of reaction is common in patients with ENL population. Higher episodes have been reported in hospital based studies. A systematic review of epidemiological data of ENL from 1980 to 2013 has reported that the frequency of multiples episodes in patients with ENL reactions ranged from 39% to 77.3% with an average of 2.6 episodes (range: 1-8 episodes). These patients experienced episodes on average at 14.35 days (range: 8-20 days) (Voorend and Post, 2013). Episodes of active ENL has been reported to last from 14.4 (Hali F et al., 2009) days to 26.1 weeks (Balagon et al., 2010). In Ethiopia, almost one third of patients with ENL reactions develop chronic condition lasting more than 2 years (Saunderson et al., 2000c). ENL often has a protracted course with episodes occurring over several more years although the majority last 12 to 24 months (Kumar et al., 2004, Pocaterra et al., 2006).

In an 11-year retrospective case record analysis of 481 outpatients with BL/LL at the Dhoolpet Leprosy Research Centre in Hyderabad, three patterns of ENL were identified: single actuate episodes, recurrent acute episodes and chronic ENL (Pocaterra et al., 2006). Acute episodes were defined as single episodes responding to steroid treatment and accounted only 6% of ENL cases, acute multiple ENL

(32%) included recurrent episodes with periods off treatment, and chronic when patients needed steroid treatment for more than six months (62%). Among 107 ENL cases identified in retrospective study in Nepal, 45% had more than one episode of ENL (Manandhar et al., 1999).

3.3.4. Pathology

An intense neutrophilic infiltrate throughout the dermis and sub-cutis is the histological feature seen in biopsies of acute ENL skin lesions. Polymorphs infiltrate the granuloma and there is vasculitis and macrophage degeneration together with the breakdown of foam cells. Tissue oedema, vessels exhibiting fibrinoid necrosis and associated vasculitis may also present. There is a local; reduction in bacterial load; most of the organisms are fragmented and granular (Post et al., 1994, Kahawita and Lockwood, 2008). As the lesion progress to chronic phase, macrophages are substituted by lymphocytes, plasma cells and histiocytes representing chronic inflammatory infiltrate. One study has reported that 36% of ENL lesions from Pakistani patients had no visible neutrophil infiltration and C-reactive protein (CRP) was eight-fold lower in these patients (Hussain et al., 1995). It has been previously reported that the timing of biopsy samples is important for an accurate picture of ENL (MEN, 1996).

3.3.5. Diagnosis

There is no definitive laboratory test for ENL. ENL is diagnosed clinically. Sudden eruptions of erythematous tender (red) papule, nodules, or plaques which may ulcerate are used as major criteria for diagnosis of ENL. Other diagnostic criteria such as mild fever, tender enlarged nerve and increased loss of sensation, oedema of extremities or face are used as supportive diagnostic criteria. Clinical diagnosis can be supplemented by histopathological features of the skin lesion. Histologically ENL is characterized by infiltration with neutrophil usually surrounding blood vessels and invading the walls. For accurate diagnosis of the disease, communication between the clinician and the pathologist is necessary as in chronic ENL lesions neutrophil infiltration may not be evident.

3.3.6. Treatment of ENL reaction

The treatment of ENL is aimed at to control acute inflammation, easing pain and preventing eye and nerve damage. The majority of ENL reactions require immunosuppression. The more severe ones require high doses of corticosteroids, usually starting with prednisolone 60mg daily (World Health Organization, 2006). The recurrent nature of the condition means that steroid-induced side effects may become a significant problem. Thalidomide 300-400mg daily has a remarkable effect in controlling ENL and preventing recurrences. However, teratogenicity and possible neurotoxicity of thalidomide may limit its wide application.

Steroids have been the main treatment for ENL since 1950s following the use of 4 to 7 days course of injections with cortisol or adrenocorticotropic hormone (ACTH) for treating leprosy reaction (Chaussinand, 1950) . However, it was also noted that reactions re-appeared when the treatment was stopped. As a result, the treatment duration and dose of corticosteroid was improved.

World Health Organization recommends a 12-weeks course of prednisolone (daily dose not exceeding 1mg/kg body weight) as effective treatment of ENL reaction. However, prednisolone it is not efficacious in preventing reoccurrences. Usually an ENL flare-up occurs when the prednisolone dose is decreased to 20-30mg per day (Sharma et al., 2004). Clinical experience suggests that higher doses of prednisolone being needed to control flare-ups. Furthermore, a considerable number of patients with ENL may become steroid dependent. The use of potent immunosuppressant like prednisolone is also potentially problematic in areas endemic for severe infectious diseases such as tuberculosis. High doses of corticosteroid treatment of patients with ENL for prolonged time has been implicated in steroid induced osteoporosis, diabetes mellitus and infections (Kahawita et al., 2008, Papang et al., 2009).

The investigation of alternative drugs with less adverse effects such as clofazimine (Groenen et al., 1986), pentoxifylline (Mekhlafi and al-Qubati, 1996), methotrexate (Gallo et al., 1996), cyclosporine, (Rodrigues et al., 2000, Lambert et al., 2016) have shown some promising hopes but not successful yet. Therefore, alternative drugs with less adverse effects for treatment of ENL should be explored.

CHAPTER 4: IMMUNOLOGY OF LEPROSY REACTIONS

4.1. Introduction

No clinical or laboratory tests can accurately predict who is most likely to develop a reaction or when it might occur. The risk factors and mechanisms responsible for each type of reactions are not yet well defined. Several studies have reported associations between immunological markers and leprosy reactions. However, the reports are inconsistent. Most of these studies involved small number of patients. Case definitions, controls and the assay methods used were not described in the majority of the studies. The progress and current status of immunological studies of leprosy reactions are reviewed in the following sections.

4.2. Immunology of Type 1 reactions

T1Rs are delayed hypersensitivity reactions that occur predominantly in borderline forms of leprosy (Job, 1994). *M. leprae* antigens have been demonstrated in the nerves and skin of patients experiencing T1Rs. The antigens are localised to Schwann cells and macrophages (Lockwood et al., 2002). *M. leprae* infection may lead to the expression of MHC II on the surface of the cells and this may give rise to antigen presentation which triggers CD4 lymphocyte killing of the cell mediated by cytokines such as TNF-α (Ochoa et al., 2001). However, the causes and mechanisms of the spontaneous enhancement of cellular immunity and delayed hypersensitivity to *M. leprae* antigens is not clearly known (Scollard et al., 2006). Increased lymphocyte proliferation in response to *M. leprae* antigens *in vitro* during type 1 reaction has also been reported (Godal and Negassi, 1973b).

Immunophenotyping studies of skin lesions from patients with T1R has shown that the number and percentages of $CD4^+$ T-cells are increased in T1R skin lesions compared to the skin lesion of borderlines. An increased TNF-α protein detectable in the skin and nerves of 14 Indian patients during T1Rs has been reported by counting the proportion of cells positively stained using anti-TNF-α mouse antibody. Immunohistochemical results were supported by TNF-α mRNA gene expression which was measured by in-situ hybridisation method (Khanolkar-Young et al., 1995).

T1Rs appear to be mediated via Th1 type cells and lesions in reaction express the pro-inflammatory IFN-γ, IL-12 and the oxygen free radical producer inducible nitric oxide synthase (iNOS) (Little et al., 2001, Venturini et al., 211). The INFR cohort study has reported increased levels of TNF-α, TGF-β and iNOS in the biopsies of 6 patients with type 1 reactions (Lockwood et al., 2011). Similarly, Madan et al. (2011a) has shown that the levels of TNF-α and IL-1β were significantly higher in T1Rs compared to PB cases. In this study, the level of IFN-γ in T1Rs was not significantly different compared to that of PB.

The cytokine mRNA genes expression study involving small number of patients (8 leprosy patients with T1Rs) has shown that IL-β, TNF-α, IL-2, and IFN-γ mRNA were significantly increased in the skin lesions of T1Rs compared to in other leprosy types (Yamamura et al., 1992). The expression of mRNA of various chemokines including IL-8, MCP-1 and CCL5 (or RANTES) were found to be higher in the skin lesions during type 1 reactions (Kirkaldy et al., 2003). It has been shown that 10 smear negative newly diagnosed BT patients with T1R had significantly elevated levels of plasma CXCL10 and IL-6 compared to BT non-reactional controls. None of these individuals had neuritis (Stefani et al., 2009).

Longitudinal immune responses and gene expression profiles study in T1Rs has shown increased production of IFN-γ, IP-10, CXCL9, IL-17A and VEGF at diagnosis of T1R compared to before T1R, whereas a simultaneous decrease in IL-10 and G-CSF was observed at T1R (Geluk et al., 2014). In this study, RNA expression profiles revealed that IFN-induced genes, vascular endothelial growth factor (VEGF), and genes associated with cytotoxic T-cell responses (GNLY, GZMA/B, PRF1) were upregulated during T1R, whereas expression of T-cell regulation-associated genes were decreased.

A Longitudinal immune profiles analyses of type 1 leprosy reactions in Bangladesh, Brazil, Ethiopia and Nepal has shown that IFN-γ-, IP-10-, IL-17- and VEGF-production by *M. leprae* (antigen)-stimulated PBMC peaked at diagnosis of type 1 reactions, compared to when reactions were absent. In contrast, IL-10 production decreased during type 1 reaction while increasing after treatment (Khadge et al., 2015). These authors have also reported that the circulating IP-10 in sera were significantly increased during type 1 reactions while humoral immunity,

characterized by *M. leprae*-specific antibody detection, did not identify the onset of type 1 reactions.

4.3. Immunology of Erythema Nodosum Leprosum

ENL reactions were initially thought to be due to immune-complex deposition in the blood vessels suggestive of Arthus reaction (Wemambu et al., 1969a). However, immune-complex deposition is not consistently demonstrable and typical features of immune-complex diseases are absent in ENL. Recent data suggest that cell-mediated immune responses may also play an important role in the pathogenesis of the disease (Laal et al., 1985 , Nath et al., 2015). In the following sections, the research findings supporting these two hypotheses are reviewed. Following the review, the strengths and weakness of these findings are accounted.

4.3.1. Immune-complex deposition as causes for ENL

An immune-complex (IC) is formed by the binding of an antibody to soluble antigens. Once the immune-complex is formed, a number of responses such as complement activation, opsonisation, phagocytosis, or processing by proteases may be initiated. Failure to clear the immune-complex results in deposition of the complexes in situ or in the circulation and it becomes detrimental to the host. Deposition of the antigen-antibody complex in tissues or in the circulation is the hallmark of human autoimmune diseases (Schmidt and Gessner, 2005) such as vasculitis (serum sickness), Systemic lupus erythematosus (SLE) and Rheumatoid arthritis (RA) (Mayadas et al., 2009).

The immune-complex aetiology of ENL was first proposed in the late 1960s, by Wemambu et al. (1969a). In this study, 17 ENL and 6 uncomplicated LL controls were included. Direct immunofluorescence was used in order to demonstrate granular deposits of immunoglobulin and complement in a perivascular distribution corresponding to the polymorphonuclear infiltration in the dermis of 10 out of 17 ENL lesions but not in those of uncomplicated LL lesions. Soluble mycobacterial antigen was found in some of (7/17) the immune-complexes. Following the observation, these authors suggested ENL as Arthus reaction. This concept was supported by the presence of circulating immune-complexes and the demonstration of mycobacterial antigens, complement and immunoglobulins around the vessels in

some lesions of patients with ENL (Wemambu et al., 1969b, Turk and Waters, 1971, Drutz and Gutman, 1973). An Arthus reaction involves deposition of immune-complexes with vasculitis and plymorphonuclear infiltrate. Others suggest that the immune-complexes are extravascular and hence, ENL is different from the Arthus reaction (Ridley and Ridley, 1983, Andreoli et al., 1985).

Following the Wemambu and his group's report, a larger number of leprosy patients (38 ENL and 13 LL) were studied to confirm the previous findings by Wemambu et.al. In this study, although, the immunoglobulin, complements and mycobacterial antigen were not detected in the lesions of LL controls, these substances were detected in less than half of the skin biopsies (21/47) taken from patients with ENL (Waters et al., 1971). In another study, nonspecific immunoglobulin deposits (IgG) were demonstrated in the dermis of all skin lesions of patients with ENL(25 ENL) but none of the 10 LL patient controls had such granular deposits (Anthony et al., 1978). They also reported that serum complement (C3) levels were decreased in 22 out of 25 patients with ENL and elevated in 8 out of 10 LL patient controls without reaction. Immunoglobulin deposits were not consistently seen in all patients with ENL in the blood vessels and in cell infiltration. Immunoglobulin deposits were seen in the blood vessels (7/25) and in cell infiltration (12/25) of ENL cases. The authors suggested that the negative fluorescence results in the blood vessels and cell infiltration in patients with ENL were due to the biopsies being taken when the lesion was subsiding.

The role of immune-complexes in the sera of patients with leprosy had been addressed by several studies in the 1970s and 1980s using different methods such as the C1q immunoassay (Moran et al., 1972), the platelet aggregation test (PAT) (Wager et al., 1978, Sehgal and Kumar, 1981), The Rajii cell binding technique and radioactive method (Geniteau et al., 1981), ^{125}I-C1q binding activity (Bjorvatn et al., 1976), Polyethylene glycol (PEG) (Park et al., 1984, Rojas and Segal-Eiras, 1997), Phenolic glycolipid-1 (PGL-1) (Andreoli et al., 1985). However, serum samples from leprosy with or without reaction showed positivity to these tests.

The presence of soluble immune-complexes was investigated in the sera of patients with LL forms by measuring the reactivity of serum samples with C1-q complement (Rojas-Espinosa et al., 1972). These authors reported that 78% (39/51)

of patients with lepromatous leprosy had immune-complexes, while only 33% (3/9) patients with BL had detectable immune-complexes. However, it was not mentioned in the study whether the patients with LL included to the study had reaction. By using a non-quantitative agarose C1-q precipitation method, Moran et al. (1972) confirmed the presence of immune-complexes in the sera of 76% of ENL cases. Bjorvatn et al. (1976) measured the serum binding activity ^{125}I-C1q in 26 Ethiopian leprosy patients (13 ENL, 7 LL without reaction and 6 TT) and found an increased immune-complexes in 80% of ENL and 82% of LL cases which was not significantly different between the two groups as opposed to the previous findings by Moran et al. (1972). However, an increased plasma C3d level was detected in 70% ENL cases but only in 18% non-reactional LL patients. This finding led to the hypothesis that ENL is caused by the extravascular immune-complexes formation. The presence of extravascular complement fixing complexes was also reported by Andreoli et al. (1985). These authors reported a remarkable reduction of circulating IgM antibodies to phenolic glycolipid-1 (PGL-1) antigen of *M. leprae* during ENL reaction. Hence, these two authors concluded that ENL is characterized by complement hypercatabolism since the level of the C3 breakdown product (C3d) in the plasma was found increased in the sera of patients with ENL which was rarely found in patients with uncomplicated lepromatous leprosy.

In contrary to the above findings, (de Azevedo and de Melo, 1966, Anthony et al., 1978) reported that the level of complements particularly C3 was reduced in patients with ENL reaction compared to the non-reactional groups. Anthony et al. (1978) postulated that the decreased level of C3 in ENL was most likely due to the utilization during the course of immune-complex formation. Similar decrease in serum complement level has been described in other immune-complex disorders such glomerulonephritis (Vernon et al., 2012) and acute lupus erythematous (SLE) (Leffler et al., 2014).

Wager et al analysed sera from 135 (88 LL, 36 TT/BT, 11 ENL) leprosy patients from Brazil and Ethiopia by the platelet aggregation test (PAT), previously suggested to be sensitive detector of IgG complexes in other immune-complex diseases (Agnello et al., 1976), and concluded that PAT is a sensitive detector of IgG specific to LL compared to TT but it does not differentiate ENL and non-

reactional LL. The PAT positivity was 54% in LL and 45% in ENL while it was 3% in TT/BT (Wager et al., 1978).

The presence of mycobacterial antigens in the immune-complexes from sera of LL patients was also demonstrated by other studies (Furukawa et al., 1982, Chakrabarty et al., 1983). A parallel increase of circulating immune-complex (CIC) and BI from TT to LL has been reported from Japanese leprosy patients (77L,19 TT and 21 BB) (Furukawa et al., 1982). A study by Rojas and Segal-Eiras (1997) precipitated immune-complexes from sera of leprosy patients to identify antibodies against PGL-1 and 10KDa heat shock *M. leprae* protein. According to this study, the highest level of IgM anti PGL-1 in CICs were belonged to the ENL patients which was significantly different to the control groups but not to LL/BL groups indicating that the presence of ICs could be a finding to both lepromatous leprosy and ENL.

Disrupted *M. Leprae* was found to activate more complement fixation than the intact one (Lahiri et al., 2008a). These authors suggested that immune-mediated events that precipitate erythema nodosum leprosum could be the activation of complements by the antibody-antigen complex formation following local inflammation.

Some studies were not focused on the quantity of circulating immune-complexes (CICs) in the sera but instead tried to isolate differences in the qualities of these isolated CICs. The ability of sera to solubilize immune precipitates *in vitro* through the complement system was studied in 62 leprosy patients (10 BT, 10 LL without reaction, 10 T1R and 32 ENL) by Ramanathan et al. (1985). The solubilizing capacity of sera from non-reactional patients were comparable with that of healthy controls (> 95% solubilisation) while sera from leprosy patients with T1R and ENL had significantly low levels of solubilisation (< 50%) (Ramanathan et al., 1985). They also reported that although the circulating immune complexes and serum C3d of these patients came down after the subsidence of reaction, their solubilisation remained consistently low during a three month follow-up period. However, Tyagi et al. (1990) reported that significant differences were not observed between the Polyethylene Glycol (PEG) precipitates from reactional and non-reactional TT/BT and BL/LL patients in their complement activating ability.

A recent study by Dupnik et al., used microarrays in isolated PMBCs from ENL cases and leprosy controls and tried to identify gene patterns of expression characteristic of ENL. Several components of the classic complement pathway showed increased expression in PBMCs from patients with ENL. Complement C1qA, B, C and the complement receptor C3AR1 and C5AR1 showed increased expression in ENL cases compared to the other leprosy controls. Imunohistochemical staining of skin lesions also showed increased fluorescent intensity of C1q in 3 ENL and 3 T1R cases (Dupnik et al., 2015).

In conclusion, Wemambu et al., able to confirmed the presence of immunoglobulin and complement in the skin of only 59% of patients with ENL (Wemambu et al., 1969b). They also reported that only 70% of the patients with immunoglobulin deposition showed the presence of *M. leprae* antigens within the immune-complexes. This study was the first study conceptualizing the aetiology of ENL. It is also the only study which attempted to show the co-localization of complement, immunoglobulin and *M. leprae* antigens in skin lesions in leprosy. The association of immune-complex deposition and ENL has been described by several studies (Table 4.1).

However, none of them proved immune-complex as the aetiology of ENL. It is still unclear if they initiate the pathogenesis of ENL or if they are simply a by-product of the real cause of the disorder. The detection of circulating ICs in infectious diseases states neither about their origin nor whether they will always elicit harmful consequences for the host (Hoiby et al., 1986). In patients with ENL as in any infectious disease, the presence of antibodies against the causing pathogen is part of the host defence mechanism against the infection. In infectious diseases where ICs are present possibly causing immunopathology, the true pathogenic mechanism should be the rate of IC clearance by the host and not the mere detection of ICs in the tissues. Furthermore, immune-complexes do not explain the synchronous (simultaneous) on set reactions at multiple sites in patients with ENL. In conclusion, evidence is not good enough to clearly support the long-standing IC pathogenesis dogma in ENL, despite the fact that numerous studies tried to address this for many years.

Table 4.1. Summary of previous immune- complex studies on leprosy

Author	Method	Sample	Patient definition	Main Findings
(de Azevedo and de Melo, 1966)	Complement activity	serum	33 TT, 37 LL, 18 ENL	decreased complement activity in ENL
(Wemambu et al., 1969a)	Immunofluorescence	Biopsy	17 ENL	Ig and complements deposition in 10/17 ENL cases and *M. leprae* antigen in 7/17 cases
(Waters et al., 1971)	Immunofluorescence	Biopsy	38 ENL,13 LL	20/38 ENL positive for complements (Cs) and Igs. none of LL were positive for Cs and Igs
Bonomo 1971	Immuno-electrophoresis	serum	6 LL	The IC in LL patients made up of IgG and IgM. Other Ig s were not detected
(Rojas-Espinosa et al., 1972)	C1q assay	Serum	511, LL, 9TT, 35 HC	Soluble IC detected in 76% LL, 22% TT, 3% HC
(Anthony et al., 1978)	Immunofluorescence Complement assay	Biopsy Serum	25 ENL 10 LL	Ig deposits seen in all dermis of ENL but Only in 7ENL the vessel wall and in 12 ENL in perivascular infiltrates. Ig deposits were negative for LL controls
(Wager et al., 1978)	PAT	serum	36TT/BT,88LL and 11ENL	PAT test couldn't differentiate between ENL and LL
(Sehgal and Kumar, 1981)	PAT immunofluorescence	serum biopsy	40 LL, 26 BL 6 ENL, 118HC	PAT positivity was confirmed in 85% LL and none in HC
(Mshana et al., 1983)	Enzyme based IHC	Biopsy	26 ENL 20 LL	3/26 ENL and 4/20 had Ig deposits 2/20 LL and 5/26 ENL had C deposit all ENL and LL had antigen
(Ramanathan et al., 1985)	IC-solubilisation	serum	10BT,10 LL 10 RR, 32 ENL, 15HC	low solubilisation of IC in in RR and ENL
(Chakrabarty et al., 1988)	IC - solubilisation	serum	16 LL, 3RR 8ENL	solubilisation capacity of sera from LL, ENL and RR reduced Solubilisation was not improved after ENL remission
(Rojas and Segal-Eiras, 1997)	PEG ELISA	serum	19 EN,13 HC 10 controls	IC associated anti-Anti PGL-1 and anti- 10KDa significantly increased in ENL
(Tyagi et al., 1990)	PEG	serum	9TT,8 BL/LL, 7RR,8 ENL, 10 HC	No significant difference between the PEG precipitate for all leprosy groups. PEG precipitate from BL/LL/ENL activated both classical and alternative pathway while that of TT/BT/RR activated only the alternative pathway
(Dupnik et al., 2015)	I immunofluorescence	Biopsy	3RR, 3ENL and 5BL	Immunohistochemical staining of skin lesions showed increased C1q in both RR and ENL

4.3.2. Neutrophils in ENL

Histologically, neutrophils are considered the signature cell in ENL lesions (Mabalay et al., 1965). Mabalay *et.al* gave a comprehensive account of the histopathology of ENL. They reported that the inflammatory infiltrate is usually seen in the deeper layers of the dermis and subcutis of ENL lesions. According to these authors, in acute lesions, within 72 hours neutrophils are the predominant cell type, whereas between 72-96 hours equal numbers of neutrophils, lymphocytes and plasma cells are seen, and along with the presence of mast cells. Chronic lesions show fewer neutrophils and eosinophils, but increased numbers of lymphocytes. Sehgal et al. (1986b) suggested vasculitis to be a major pathological event in ENL reactions, along with interstitial oedema, and degenerative and necrotizing changes seen in classical vasculitis. On the other hand, recent findings claimed that the classical signs of vasculitis are not always present in ENL (Adhe et al., 2012b).

In a study in Pakistani patients, neutrophils have been detected in only 64% (29/45) of biopsies but the biopsies were done within 7 days of appearance of lesions (Hussain et al., 1995). A retrospective study of 64 Indian leprosy patients (22 RR and 42 ENL) confirmed the presence of neutrophils within the granuloma in all ENL cases (Adhe et al., 2012b). They also reported that neutrophilic panniculitis was commonly seen in 66% (30/45) of ENL lesions. However, a cross-sectional study on the histological features of leprosy reactions in Indian patients by Sarita et al. (2013) attested the histological evidence of neutrophil infiltration only in 57% (8/14) ENL cases and neutrphilic vasculitis in 7% (1/14).

It has also been shown that the apoptosis of neutrophils is significantly increased in patients with ENL which indicates the involvement of neutrophils during ENL reactions (Oliveira et al., 1999). It is postulated that *M. leprae* and *M. leprae* derived lipoarabinomannan (LAM) could stimulate neutrophils which in turn secrete TNF-α and IL-8. IL-8 contribute to the recruitment of lymphocytes to the lesional sites which further increases TNF-α production at the site of ENL lesion and hence, tissue damage (Oliveira et al., 1999). However, at present, it is unclear if more significant role is associated with neutrophils in the pathogenesis of ENL.

The mechanisms of neutrophil recruitment at the site of disease was investigated by Lee et al. (2010). The gene expression profile of ENL lesions comprised an

integrated pathway of TLR2 and Fc receptor activation, neutrophil migration, and inflammation. According to these authors the pathway includes: i) FcR or TLR2 induction of IL-1β release; ii) endothelial activation, including the upregulation of E-selectin and subsequent neutrophil binding; iii) upregulation of inflammatory mediators associated with both neutrophils and monocytes/macrophages. Thalidomide, which is a highly effective drug in the treatment of ENL, is known to reduce neutrophil infiltration in lesions and targeted individual events in this inflammatory pathway (Lee et al., 2010).

4.3.3. The role of B-cells in ENL

B-cells enable the antigen-specific humoral immunity by forming highly specific antibodies during primary immune response. Although B-cells are traditionally known as precursors for antibody-secreting plasma cells, they may also act as antigen-presenting cells (APC) and play a role in the initiation and regulation of T and B cell responses (Martin and Chan, 2006, Hampe, 2012). The pathogenic roles of B-cells in autoimmune diseases occur through several mechanistic pathways that include autoantibodies, immune-complexes, dendritic and T-cell activation, cytokine synthesis, chemokine-mediated functions, and ectopic neolymphogenesis (Hampe, 2012).

B -cells are the least studied immune cells in leprosy and leprosy reactions. Little evidence of an increase in B-lymphocytes in ENL lesions is available. An increased percentage and absolute count of B-cells in the sera from patients with ENL has been reported (Sehgal et al., 1986b), but normal numbers of circulating B-cells have also been reported (Rao and Rao, 1986). A study looking at T-cell phenotypes in ENL lesions showed that there is no increase in B-cells (Narayanan et al., 1984). In a prospective cohort study of 13 untreated patients with acute ENL reaction, polyclonal IgG1 antibody synthesis was elevated compared to patients with stable lepromatous leprosy and decreased after the disease had subsided. However, the concentration of polyclonal IgG2 had revealed the reverse trend: decreased before treatment and increased after treatment (Kifayet et al., 1996). These authors also investigated the frequency of antibody secreting B-cells in the blood compartment of these patients with the Enzyme-Linked ImmunoSpot (ELISPOT) and found that

the decrease in *M. leprae* specific IgG1 antibody was not related to the down-regulation of B-cell reposes.

The role of B-cells in the pathogenesis of autoimmune disorders such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) is now being re-examined (Martin and Chan, 2006). It would therefore be interesting to examine the role of B-cells in the pathogenesis of ENL.

4.3.4. T- cells in ENL

There are several pieces of evidence for increased T-cell activity in leprosy patients with ENL reactions in comparison with those with non-reactional LL patients. The involvement of cell-mediated immunity in the pathogenesis of ENL was first postulated by Rea et al. (1972). They noted that patients with ENL reactions have apparently normal cell-mediated immunity compared to those with non-reactional LL whose cell-mediated immunity is impaired. In 1982, it was hypothesized that ENL is precipitated by an imbalance of T-lymphocyte subpopulations (Mshana et al., 1982). According to the hypothesis, ENL has two phases: initiation, due to an imbalance in T-cell subpopulations with decreased suppressor cells and perpetuation. The analysis of tissue lesions of ENL by *in situ* hybridization technique suggested that ENL reactions may be visualized as transient reduction of cytotoxic ($CD8^+$) T-cells activity, leading to partial augmentation of Th ($CD4^+$) activity perhaps sufficient to result in antibody and immune-complex formation, but insufficient to clear bacilli from lesions (Cooper et al., 1989).

A direct evaluation of thymus-dependent lymphocytes of peripheral blood in leprosy patients with ENL reactions by rosette assay method has shown an increased percentage and numbers of T-cells in patients with ENL reactions compared to non-reactional lepromatous leprosy patients (Lim et al., 1974).

An evidence for transient enhancement in T- cell functions was also reported during the acute phase of ENL reactions in Indian patients (Laal et al., 1985). Laal et al, assessed the status of T-cell mediated functions in 13 non-reactional LL and 15 patients with ENL reactions. They found that all non-reactional LL patients showed lack of antigen-induced leukocyte migration inhibition and lymphoproliferative *in vitro* response to soluble and integral *M. leprae* antigens. These patients were also

showed poor mitogen response. However, patients with active ENL reaction had strong antigen-induced leukocyte migration inhibition and enhanced mitogen induced lymphoproliferation. In the same study, T-cell subsets in PBMCs from ENL and LL were characterized by indirect immunofluorescence using monoclonal antibodies; OKT3, OKT4 and OKT8. Patients with ENL reactions had lower OKT4/OKT8 ratio compared to non-reactional LL patients (Laal et al., 1985).

Rao and Rao (1986) measured the cell-mediated immune responses in 77 Indian leprosy patients (44 ENL and 39 LL). Patients with ENL reactions had significantly enhanced *M. leprae* specific immune responses with increased percentage of early T-lymphocytes (47.68%) in ENL compared to LL (38.96%) patients. However, there was no response to the lepromin skin test in patients with ENL reactions in contrast to the enhanced *in vitro* cell-mediated immune responses (Rao and Rao, 1986). These authors also found that the number of T-lymphocytes in the post-ENL remained high (45.66%) compared to the non-reactional LL patients. On the other hand, Rea et al, reported that the proportions of T-cell subsets in ENL and LL showed no difference in a study involved 122 leprosy patients (43 LL, 23 ENL, 13 RR, 13 BL and 31 TT); 22 healthy controls and 27 patients with SLE (Rea et al., 1984).

The numbers and distribution of T-lymphocyte subsets in the lesions of reactional states of leprosy (6 RR, 9 ENL and 2 Lucio's reactions) with those of stable, non-reactional patients (6 TT, 2BL and 7LL) was investigated using monoclonal antibodies and immunoperoxidase technique by Modlin et al. (1983) . In this study, it has been shown that the T-helper/suppressor ($CD4^+/CD8^+$) ratio was significantly higher in ENL cases (2:1) compared to the non-reactional controls (0.7:1) (Modlin et al., 1983). According to these authors, the increased T-helper cells in ENL lesions compared to LL lesions suggests that cell mediated immune response may be important in the pathogenesis of ENL in either permitting productions of antibodies critical to immune-complex formation or as a cell-mediated immune response. A similar result was also reported from Indian study by Narayanan et al. (1984).

Bhoopat et al. (1991) studied the subsets of T-cells in the cutaneous leprosy lesions of 19 patients with acute ENL, 38 chronic ENL and 61 active non reactional LL

patients in suction-induced blisters. They found that lesions in chronic ENL had a decreased number of $CD8^+$ T-cells and increased helper/cytotoxic ($CD4^+/CD8^+$) ratio as compared to those in acute ENL and non-reactional LL patients. In the same study, systemic administration of corticosteroids was associated with a reduction of intralesional $CD4^+$ T-cell population but did not change $CD8^+$ T-cell population. The authors concluded that spontaneous lymphocyte activation *in situ*, primarily of decreased $CD8^+$ T-cells and relatively increased $CD4^+$ T-cells, are important features of chronic ENL reactions and may be an intermittent or cyclic phenomenon during the reaction. Martiniuk et al. (2012) highlighted that the possibility of up regulation of Th1 and Th17 cells during ENL reactions and after thalidomide treatment in a study conducted on 7 ENL patients in Nepal.

In conclusion, there are in consistent reports on the number of helper ($CD4^+$) and cytotoxic ($CD8^+$) T-cell phenotypes in patients with ENL reactions (Table 4.2). Several studies (Modlin et al., 1983, Bach et al., 1983, Wallach et al., 1982, Mshana et al., 1983, Narayanan et al., 1984) have reported increased percentage of $CD4^+$ T-cells and reduced $CD8^+$ T-cells with an increased $CD4^+/CD8^+$ ratio in patients with ENL reactions compared to patients with non-reactional lepromatous leprosy. In contrast to these findings, Laal et al. has reported a reduced $CD4^+/CD8^+$ ratio and increased percentage of $CD8^+$ T-cells in patients with ENL reactions compared to patients with LL (Laal et al., 1985). Van Voorhis et al. (1982) and Rea et al. (1984) on the other hand, concluded that there is no significant percentage difference between helper and suppressor cells, as well as helper/suppressor ratio in patients with ENL reactions and non-reactional LL.

The phenotypic and functional roles of different subsets of T-cells in the pathogenesis of ENL is not clearly delineated. Although several attempts made to define the role of T-cells in ENL, they suffer from lack or poor study designs. Case definitions and proper controls are not included in most studies. Most of these studies were cross-sectional studies. However, cross-sectional studies provide little information on the kinetics of T-cells during the course of ENL reaction. In the current study, we included considerably a large number of untreated ENL cases and LL patient controls with follow-up of for at least 28 weeks.

Table 4.2. Summary of previous T- cell studies in ENL*

Authors	Description of the study	Number of patients	Methods used in the study	Major findings
(Lim et al., 1974)	The absolute number and the proportion of T lymphocytes in patients with ENL and LL were described.	ENL=7 Others= 29	Rosette assay	High %T cells in ENL than in LL
(Rea et al., 1984)	The proportion of T lymphocyte subsets using monoclonal antibodies directed against $CD4^+$ and $CD8^+$ T cells in PBMCs of patients with ENL and LL were described.	ENL=19 Others =103	Flow cytometry	The % T cells in ENL and LL is the same
(Modlin et al., 1983)	The numbers and distribution of T lymphocyte subsets in the tissues of ENL, LL and TT patients were determined and compared.	ENL=9 Others=8	Immunopero xidase	Increased $CD4^+/CD8^+$ ratio in ENL compared to LL
(Laal et al., 1985)	The antigen -induced leukocyte migration inhibition and antigen-induced lymphoproliferation of lymphocytes in stimulated PBMCs of patients with ENL and LL were measured	ENL=15 LL=13	Leukocyte migration inhibition and Immunofluor escence	Decreased $CD4^+/CD8^+$ ratio in ENL compared to LL
(Yamamura et al., 1992)	Th1 and Th2 associated mRNA expression of cytokines were quantified	ENL=3 RR=2	PCR	ENL is associated with Th2 cells
(Goulart et al., 2000)	Quantified level of TGF-β1 production in PBMCs of ENL and LL cases	ENL=5 others=13	ELISA	ENL is associated with Th2 cells
(Nath et al., 2000)	Th1 and Th2 associated cytokines production in PBMCs of patients with ENL and LL were measured	ENL=8 LL=8	RT-PCR	ENL is associated with Th1 cells

*Only representative articles included.

4.3.5. Th-17 in ENL

The inflammatory process of tissue damage is not governed by a single cytokine or a subset of T-cells. It is a complex phenomenon that involves the interaction of various cells. During the era of Th1/Th2 functional classification of T- helper cells, it was believed that Th1 cells drive cell-mediated immune response leading to tissue damage and Th2 cells derive antibody mediated response mainly in allergic reaction. However, the mechanisms of T- cell mediated tissue damage could not explained fully by the Th1/Th2 hypothesis which led immunologists for further investigation of the Th1/Th2 paradigm in inflammation and tissue damage (Steinman, 2007).

Th17 cells have been identified as a new subset of the T- helper cells and as potential mediators of inflammation associated with various autoimmune and mycobacterial diseases (Pandhi and Chhabra, 2013). Th17 cells are the least studied T-cells in leprosy and only two studies have indicated the involvement of Th-17 in immunopathogenesis of ENL (Martiniuk et al., 2012, Attia et al., 2014, Saini et al., 2016).

4.3.6. Regulatory T cells in ENL

It has previously described that regulatory T-cells (Tregs) inhibits naïve $CD4^+$ T-cell proliferation and differentiation, prevent cytotoxic activity of $CD8^+$ T-cells, supress the activation and antibody production of B-cells, and limit the stimulatory capacity of antigen presenting cells by down regulating the surface expression of costimulatory molecules such as CD80 and CD86 (Shevach, 2009) (Figure 4.1).

Reduced percentage of Tregs has been associated with immune-complex mediated autoimmune diseases such as Wegener's granulomatosis (WG) (Morgan et al., 2010) and Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (Marinaki et al., 2005, Wilde et al., 2010). In patients with these diseases, the percentage of Tregs is inversely related to the disease relapse and in active patients a relatively increased proportion of Tregs is associated with rapid disease remission. However, the role of Tregs in leprosy has only been addressed by few studies (Attia et al., 2010, Attia et al., 2014, Bobosha et al., 2014b, Saini et al., 2014a, Saini et al., 2016) and a clear picture has not arrived at yet.

Attia et al. investigated the frequency of circulating Tregs in different clinical forms of Egyptian leprosy patients (9 TT, 8 LL, 6 ENL, 2 RR and 13 intermediates). The analysis of PBMCs by flow cytometry showed that both the absolute count and percentage of Tregs were significantly lower in patients with ENL reaction (1.2%) compared to patients with LL 2.8% (Attia et al, 2010). According to these authors the median Tregs/Teffs ratio was significantly lower in patients with ENL (0.08) compared to patients with LL (0.22). Surprisingly, they also reported that significantly higher percentage of Tregs-FoxP3 expression along with higher percentage of effector T-cells in patients with ENL compared to the other forms of leprosy. It is logical that effector T-cells (Teffs) and regulatory T- cells have inverse relationship. When one is upregulated, the other one is downregulated. Hence, one could not expect increased Teffs and Tregs in parallel in ENL reaction.

Recently, the same authors investigated the levels of IL-17, TGF-β and IL-10 and Tregs in 43 untreated Egyptian leprosy patients (6 TT, 5 PNL, 9 BB/BT/BL, 11 LL, 6 RR, 6 ENL) and 43 healthy controls (Attia et al., 2014). They reported that the production of cytokines IL-10, TGF-β, IL-17 were not statistically significantly different in the patient groups. Significantly highest percentage of Tregs was obtained in patients with TT leprosy (5.8%) compared to LL (2.8%) and ENL (1.15%) which contradicts with the findings of 2010 by the same author. It is very strange to expect highest percentage of Tregs in TT as compared to LL. Because patients with TT leprosy have strong cell-mediated immune response while those on the LL pole are characterized by unresponsive cell-mediated immunity. Several independent studies have shown that patients with LL had significantly the highest percentage of Tregs compared to the other leprosy forms of the spectrum (Palermo et al., 2012, Bobosha et al., 2014b, Saini et al., 2014a, Parente et al., 2015).

A recent study performed flow cytometry in PBMCs isolated from 6 patients with ENL in comparison to 8 LL patient controls, after stimulation with *M. leprae* sonicated antigen (MLSA), and described a significant reduction of percentage of $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs and Mean Fluorescence Intensity of FoxP3 in PBMC of ENL patients (Saini et al., 2016). However, the same study also reported an increased expression of FoxP3 in the PBMCs of patients with ENL comparted to LL controls by qPCR. Small sample size, in consistent flow cytometry gating or

lack of positive and negative template controls during RNA extraction, cDNA synthesis and qPCR amplification could contribute to the observed difference.

The discrepancy in the current study could be due to lack of patient definition, confounding effects in statistical tests or lack of appropriate control for each group. For example the level of IL-17 production in patients with LL was 12.5pg/ml while in patients with ENL it was 20.5pg/ml but statistical test did not performed to compare both groups. Furthermore, the definition of effector T-cells and information on the cellular markers used to measure effector T-cells were not given by the authors for further comment. Sometimes, T-cells other than Tregs may be considered as effector T-cells although in fact these non-Treg cells include effector, memory and naïve T cells. Particularly naïve T-cells should not be categorized with the effector T-cells group as their biology is completely different.

An immunohistochemical study of 96 patients with leprosy which included 2 ENL, 8 T1Rs and 86 other clinical forms of leprosy has shown the expression of FoxP3 in the skin lesions of all clinical forms of leprosy with an average density of 2.82% of the infiltrate (Parente et al., 2015). FoxP3 positive cells were observed inside and around the granulomas in TT and BT lesions where as in ENL and LL lesions they were found randomly in the diffuse macrophages infiltrate. Significantly increased percentage of FoxP3 positive staining was found in T1R but significant difference was not observed between the other clinical forms including ENL (Parente et al., 2015).

In conclusion, it seems that both the percentage of Tregs and the balance between effector T-cells (Teffs) and Tregs (Teffs/Tregs) ratio may play role whether patients with LL remain stable or proceed to ENL reaction. The difference in Tregs and Teffs ratio rather than differences in absolute numbers of Tregs and Teffs influence the outcome of the infection as reviewed by Belkaid and Rouse (2005). In autoimmune diseases, the imbalance of activated T- cells versus Tregs is considered as a key factor for the immmunopathogenesis of these diseases (Marinaki et al., 2005, Morgan et al., 2010).

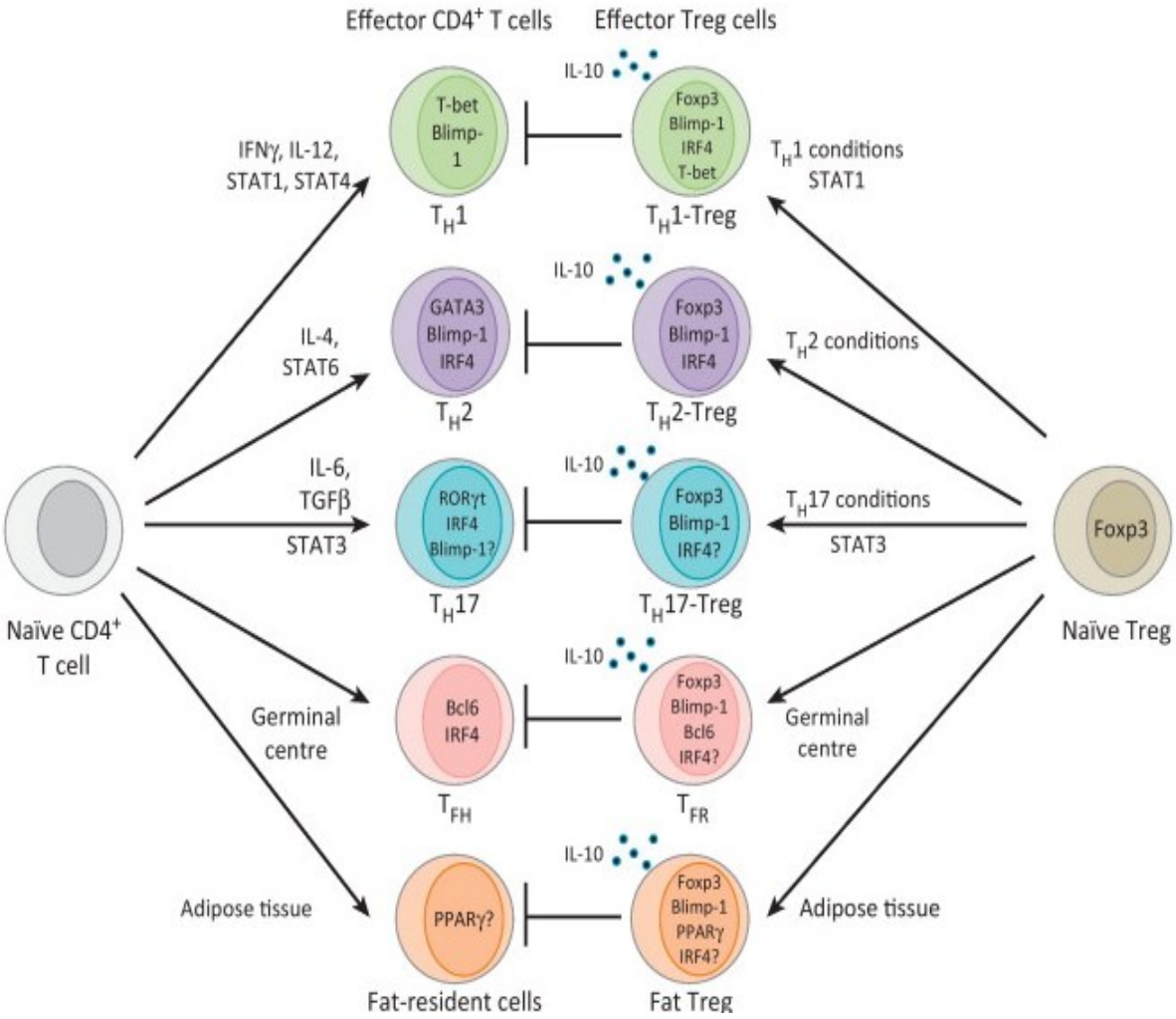


Figure 4 .1. Differentiation of effector $CD4^+$ T cells and regulatory T (Treg) cells (Cretney et al., 2013).

4.3.7. Cytokines in ENL

In leprosy the cytokines research focused mainly on the association of differential cytokine profile with the spectral pathology of the diseases. However, the results from these various studies have been varied and conflicting. The presence of both Th1 and Th2 type cytokines in the pathogenesis of ENL has been detected from the lesions and sera of patients with ENL reaction (Sreenivasan et al., 1998, Moraes et al., 2000, Teles et al., 2002). Th1 like cytokine pattern was found in the sera of 85% of patients with RR and 64% ENL with basal expression of INF-γ (Sreenivasan et al., 1998). The presence of INF-γ and absence of IL-4 was taken as indicative of Th1 pattern while the reciprocal pattern as of Th2 by these authors.

Similarly INF-γ was detected in 84% of ENL and 100% of T1R in the sera of Brazilian patients (Moraes et al., 1999b). The absence of IL-4 in lesions of ENL was reported by Nath et al. (2000), which suggests that ENL is associated with Th1 type reaction than Th2 type. On the contrary, Goolart et al., reported that ENL reaction elicited a Th2 type response with the production of IL-6, IL-8, IL-10 and TNF-α whereas T1R showed mainly Th1 type response with the releases of IL-1β, TNF-α, IL-12 and INF-γ (Goulart et al., 2000). Fink et al., suggested that there is no clear association of a clinical form of leprosy with either Th1 or Th2 cytokine secretion profile in PBMCs of leprosy patients (Fink et al., 1996).

High levels of IL-6 was observed in patients with ENL than in T1R and the levels of IL-6 was found to be positively correlated with bacilli index (Belgaumkar, 2007). Barnes et al., reported that TNF-α production in the PBMCs of patients with ENL reaction was significantly higher compared to patients with T1R and LL which was significantly reduced upon thalidomide administration (Barnes et al., 1992b). On the other hand, a study in 131 Indonesian leprosy patients among which 44 ENL, 5T1R and 68 LL/BL included, no significant difference in IL-6 and TNF-α levels was observed between ENL cases and non-reactional LL patients (Iyer et al., 2007). However, they found that INF-γ and soluble IL-6 (sIL-6) were significantly elevated in serum of patients with ENL reaction compared to patients with non-reactional LL which were declined significantly after corticosteroid treatment of patients with ENL (Iyer et al., 2007). On the contrary, a Mexican study which included only 9 ENL, 6 non-reactional LL/BL and 4 TT/BT patients reported

that serum IFN-γ was significantly lower in patients at the onset of ENL and was increased after 1 and 2 months of thalidomide treatment while serum TNF-α was significantly higher in the patients that developed ENL compared to those that did not develop the reaction (Sanchez et al., 1998).

In T1Rs IL-1β, TNF-α, IL-2 and INF-γ mRNA were prominent and found to be increasing during the course of the reaction parallel with decreasing the expression of mRNA for IL-4, IL-5 and IL-10 (Yamamura et al., 1992). On the other hand, in ENL reactions selective increase in expression of mRNA for IL-6, IL-8, and IL-10 was found with persistent expression of IL-4 and IL-5 mRNA (Yamamura et al., 1992). These authors concluded that T1R and ENL reactions are associated with type 1 and type 2 cytokine patterns respectively. The level of IL-6 and IL-6 mRNA expression was found to be higher in patients with ENL compared to in patients with LL or BL (Yamamura et al., 1992) which is not supported by the findings of Iyer et al., (2007). According to cooper et al., highest expression of INF-γ mRNA expression was seen in the cells of patients with T1Rs but in few cells of patients with ENL (Cooper et al., 1989). However, the INF-γ mRNA expression in the ENL lesions was found to be 5 times higher than in the lesion of non-reactional lepromatous leprosy patients (Cooper et al., 1989).

Higher production of TGF- β was found in patients with ENL reaction compared to T1R and non-reactional LL patients (Goulart et al., 2000). The same authors proposed that the high production of TGF-β in patients with ENL mediate a suppressive action locally, associated with the presence of PGL-1,or/and induce pro-inflammatory effects when secreted systemically by monocytes, thereby acting as a modulatory cytokine in the acute inflammatory reactions of ENL and associated with the Th2 immune response in multibacillary forms of leprosy.

IL-6 is an immunosuppressive cytokine which inhibits macrophage mediated intracellular pathogens killing. It is also known to promote B-cell responses and augment antibody formation, thereby potentiating immune-complex formation (Ochoa et al., 2010). IL-6 and type-2 cytokines would be expected to promote antibody production and suppress T-cell response. IL-6 is a strong neutrophil chemo-attractant that could augment immediate hypersensitivity character of ENL whereas IL-10 suppresses T-cell response and macrophage function whose net

effect of these cytokines would be to the favour of continuation of infection (Yamamura et al., 1992). The increase of IL-12 in patients with ENL suggests that the possibility of upregulation of both Th1 and Th-17 during ENL development. This indicates that Th-17 could be involved in the pathogenesis of ENL (Martiniuk et al., 2012).

The slightly elevated levels of INF-γ in patients with ENL reactions as compared to patients with non-reactional BL and LL pinpoints the possible role of cell mediated immunity in the pathogenesis of ENL according to Moubasher et al. (1998a). However, these data were generated from only one patient with ENL reaction and hence, needs confirmation in larger sample size.

Table 4.3. Summary of previous cytokine studies in ENL

Author	Lab method	sample	Patient definition	Conclusion	Comment
(Madan et al., 2011c)	ELSA	Serum	Untreated 61 Leprosy cases (38PB, 13MB,6 NP,6RR and 4ENL) Case definition: not given	IFN-γ increased in ENL compared to LL IL-10, TNF-α and IL-1β same in LL and ENL IL-10 increased in ENL compared to RR but IL-1β and IFN-γ similar in both groups	Mean instead of median was used for statistical test for skewed data and type II error most likely affected the result*
(Sehgal et al., 1986b)	ELSA	serum	11 RR, 11ENL	The level of soluble IL-2 receptor in ENL and RR are not significantly different	The analysis is not clear. P values not given in the table. Patient treatment status unknown.
(Attia et al., 2014)	ELISA, Flow-cytometry	Serum	Untreated (6TT,5PNL ,11LL,6 RR ,6 ENL and 9 Borderline,	Level of IL-10, TL-17,TGF-β production the same in LL and ENL IL-22 increased in ENL	Small sample size. Chi-square test were used and mean/median comparison was not performed
(Sanchez et al., 1998)	ELISA	Serum	9 ENL,6LL, 4BT/BL,10 HC	High TNF-α and Low INF-γ at onset of ENL TNF-α decreased and INF-γ increased during thalidomide treatment	The duration of ENL in patients ranging from 1 to 30 years. Patients are heterogeneous
(Belgaumkar, 2007)	ELISA	serum	1ENL,5RR others 94	INF-γ & IL-6 are high in T1R and ENL respectively	The conclusion is made based on only 1 ENL patient
(Iyer et al., 2007b)	ELISA	serum	34 LL,78BL,3 BB, 6BT,10TT, 44ENL,5R R	High INF-γ and sIL-6R produced in ENL than in LL/BL IL-4, IL-10, IL-6,TNF-α and Neopterin levels same between ENL and LL/BL	Conclusion is made based on 17 ENL, 1 T1R and 68 LL. Possible bias from loss to follow-up.

Author	Lab method	sample	Patient definition		Conclusion
(Nath et al., 2000)	RT-PCR	PBMCs	8 ENL,1BL 7LL	IL-4 and IL-10 gene expressions higher in LL compared to LL and that of 12p40 and INF-γ higher in ENL	Small sample size but good experimental design
(Moraes et al., 1999b)	RT-PCR	PBMCs and biopsy	20ENL, 11RR, 17LL/BL,	mRNA for IL-6,IL-10,IL-12,TNF-α,IFN-γ upregulated both in RR and ENL while IL-4 down regulated in both groups	Statistical analysis were not done. The Presence and absence of these genes in the sample was taken as comparison methods.
(Stefani, 2009)	Cytokine –array kit	Plasma	10RR,10ENL, 10PB,9 MB	Ip-10 and IL-6 elevated in RR ; IL-7 and IL- 6 elevated in ENL compared to their respective controls ,	Small sample size for nested case-control study.

*The mean of TNF-α for LL was 50pg/ml with Sd 76.78pg/ml for ENL mean 105.45pg/ml with Sd 56.78pg/ml. However a p value 0.2 was given. In LL cases Sd is > mean. Hence the data is not normally distributed. Using mean for comparison increases type II error.

4.3.8 Conclusion on the immunology of ENL

Reduced percentage of Tregs has been associated with immune-complex mediated autoimmune diseases such as Wegener's granulomatosis (WG) (Morgan et al., 2010) and Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (Marinaki et al., 2005, Wilde et al., 2010). In these patients, the percentage of Tregs is inversely related to the disease relapse and in active patients a relatively increased proportion of Tregs is associated with rapid disease remission. However, the role of Tregs in leprosy has been addressed only by few studies and no definitive conclusions have been made.

The generation and the fate of Tregs depend on the type and availability of specific cytokines, i.e. their differentiation, expansion and function is directed by the microenvironment surrounding them. The development and maintenance of Tregs depend on TGF-β (Wang et al., 2011). IL-2, the receptor for CD25, plays an important

double-edged role in both enabling Tregs to suppress to Teffs as well as for the activation and clonal expansion of Teffs (Corthay, 2009). At the onset of ENL reaction, high production of TNF-α has been repeatedly reported by various investigators (Barnes et al., 1992b, Memon et al., 1997, Sampaio and Sarno, 1998, Sampaio et al., 2011). Increased concentration of INF-γ and IL-6 in sera of patients with ENL compared to patients with non-reactional LL were previously reported (Belgaumkar, 2007, Iyer et al., 2007).

It has been shown that TNF-α downregulates human regulatory T cells (Ehrenstein et al., 2004, Valencia et al., 2006) in active Rheumatoid arthritis (RA) patients which was restored by treating these patients with anti-TNF- antibody (infliximab). The dysfunction of Tregs by TNF-α correlates with increased expression of IL-17, INF-γ and TNF-α controls the balance between Tregs and pathogenic Th17 and Th1 cells in the synovium of individuals with rheumatoid arthritis through FoxP3 dephosphorylation (Nie et al., 2013). Toll like receptors on antigen presenting cells induce IL-6 upon recognition of microbial products and IL-6 suppress Tregs (Pasare and Medzhitov, 2003) in favour of the induction of T-cells activation *in vivo*.

By applying the same scenario, it is possible to hypothesize that activation of T-cells are likely to initiate ENL reactions which can be further complicated by the induction and formation of immune-complexes. This hypothesis is supported by the presence of strong antigen induced leukocyte migration inhibition and enhanced mitogen induced lymphoproliferation in patients with ENL reactions unlike in patients with non-reactional LL as previously described. Furthermore, the pro-inflammatory cytokines such as TNF-α, INF-γ and IL-6 are frequently elevated in ENL reactions. It has been confirmed that these cytokines suppress Tregs in other diseases such as WG, ANCA-associated vasculitis, and RA. The detection of these pro-inflammatory cytokines in patients with ENL reactions support the proposition that ENL reaction is driven by the imbalances between T-lymphocytes. In several immune-complexes mediated diseases such as RA, SLE, WG, ANCA-associated vasculitis and others the imbalance between Tregs and Teff cells is reported as the underlying causes of these diseases. Therefore, since ENL is considered as an immune-complex associated disease it is likely that the imbalance between these T-lymphocytes play role for the initiation and progression of the disease.

However, the immunopathogenesis of ENL is further complicated by the heterogeneous condition of the disease (acute, recurrent or chronic). These conditions of ENL might be correlated with the varying immune profile in these patients. In addition to the limited number of studies available regarding ENL, previous studies didn't consider the possible variation in immune profile between the various clinical conditions of ENL. Conclusions in previous studies were drawn from either cross-sectional studies or case studies and they lack proper study design. Proper case definitions and control selection criteria are not mentioned in most of these previous studies. Therefore, we hypothesized that erythema nodosum leprosum reaction is associated with increased percentage of activated T-cells due to decreased frequency of regulatory T-cells and hence aimed at to describe the relative percentages and phenotypes of T-cells in patients with ENL before and after treatment compared to lepromatous leprosy patient controls.

In our case-control follow-up study, we set criteria for ENL definition and LL patient controls with follow-up at least for 28 weeks. Clinical and biological samples were obtained three times from each patients. We investigated several immune factors such as T- cells, memory T-cells, regulatory T-cells, B-cells, memory B-cells, pro-inflammatory cytokines or chemokines (IL-6, IL-8, IL-17A, IFN-γ, IL-1β, TNF-α, TGF-β) and their gene expressions both in the blood and in lesions and anti-inflammatory cytokine (IL-10). These findings were also supplemented by histological and clinical data. Hence, we believe that this comprehensive data together with our large sample size, provide strong evidence as to the immune-profiles of ENL reaction which can be used as a base line for further studies.

CHAPTER 5: MATERIALS AND METHODS

OVERVIEW

This chapter presents a description of the materials and methods used in the study. It is presented in 6 sections for clarity since several laboratory procedures are involved. In section 1, the patients and the study set-up are discussed. An overview of the study design including the description of matched case-control study as a research design and its relevance to this study is discussed. Study site description, sample size determination, inclusion and exclusion criteria, sampling frame, demographic and clinical data recording methods are described in this section. Section 2 deals with clinical sample collection and processing. Clinical sample collection and processing such as PBMCs isolation, freezing of cells and storage conditions are described in section 2. Section 3 describes flow cytometry. Basic principles of flow cytometry are briefed in this section. Panel construction methods for regulatory T-cells, memory T-cells and B-cells are described in this section. List of fluorochrome-conjugated antibodies for each panel and the sources of each antibody are given. Antibody titration method, staining cells for flow cytometry work, flow cytometry settings, data acquisition and the gating strategy for each panel are described in this section. Section 4 deals with ELISA. A brief description of the principles of ELISA is given. Lymphocyte stimulation methods, harvesting supernatant for ELISA, ELISA procedures and ELISA data recording methods are described. In section 5, the real-time PCR assay methods are described. Protocols for RNA isolation from PAXgene Blood RNA Tubes and RNAlater stored skin biopsies are described. Protocols for cDNA synthesis, primer design, choice of the housekeeping gene (control gene), optimization of quantitative PCR and methods for obtaining the relative gene expression are described in this section. Section 6 describes all about the statistical methods. Data recording and management, data analysis strategy used for each section and data presentation methods are described in this section.

SECTION 1: THE PATIENTS AND STUDY SET-UP

5.1.1. Case definitions

Clinical case definitions (Pocaterra et al., 2006)

The clinical assessment of the patient was used as main diagnostic criteria for cases (lepromatous leprosy patients with ENL reaction) and controls (lepromatous leprosy patients without ENL reaction).

1. **Erythema Nodosum Leprosum (ENL)**

   ENL was clinically diagnosed when a patient had tender subcutaneous erythematous skin lesions and systemic features of disease often fever, neuritis and bone pain occurring in patients with lepromatous leprosy or borderline lepromatous leprosy. Other accompanying systemic features included neuritis, joint pain, bone tenderness, orchitis, iritis, oedema malaise, anorexia and lymphadenopathy.

2. **Lepromatous leprosy (LL)**

   Lepromatous leprosy was clinically diagnosed when a patient had widely disseminated nodular lesions with ill-defined borders and BI above 2.

3. **Acute ENL**

   Acute ENL was defined as an ENL episode lasting less than 24 weeks of prednisolone treatment

4. **Chronic ENL**

   Chronic ENL was defined as an ENL occurring for 24 weeks or more during which a patient has required ENL treatment either continuously or where any treatment free period has been 27 days or less.

5. Recurrent ENL

Recurrent ENL was defined as a second or subsequent episode of ENL occurring 28 days or more after stopping or steady decrease of steroid treatment for ENL.

6. ENL recurrence or flare-up

ENL recurrence or flare-up **w**as defined as the appearance of new ENL nodules after initial control, either whilst on treatment or after 28 days off treatment.

7. New ENL case

New ENL case was defined as the occurrence of ENL for the first time in a patient with lepromatous leprosy**.**

Histological definitions (Lockwood et al., 2011).

The histological assessment of paraffin-embedded patient biopsy sample was used as supplementary confirmation for the definition of cases and controls whenever necessary.

1. ENL

When a polymorphonuclear neutrophil infiltrates on the background of a macrophage granuloma which can be accompanied by oedema, vasculitis and/or panniculitis is seen in H & E stained sections of biopsy, it was recorded as erythema nodosum leprosum case.

2. LL

When macrophages and foamy cell collections are present with numerous bacilli interspersed with a sparse number of lymphocytes in H & E stained sections of biopsy, it was recorded as lepromatous leprosy case.

5.1.2. The study area

This study was conducted at AHRI/ALERT campus, Addis Ababa, Ethiopia. Patients were recruited at Red Medical Clinic (RMC) of ALERT Hospital. Clinical sample processing such as peripheral blood mononuclear cells (PBMCs) isolation, storage, flow cytometry staining and reading, ELISA and qPCR have been done at Armauer Hanssen Research Institute (AHRI).

ALERT: It was initially the All Africa Leprosy Rehabilitation and Training Centre (hence the acronym), but the official name is now expanded to include tuberculosis:

All Africa Leprosy and Tuberculosis Rehabilitation and Training Centre. ALERT is the only leprosy referral centre in Ethiopia. It was established by Dr. Thomas Lambie, an American missionary doctor in 1922. It was named as the princess *Zannaba Wark* Hospital by the name of the Emperor Haile Selassie's daughter in 1966. Leprosy patients are seen at Red Medical Clinic (RMC). RMC is a special clinic within the hospital dedicated for leprosy, diagnosis, treatment and rehabilitation.

AHRI: Armauer Hansen Research Institute (AHRI) is a biomedical research institute located in ALERT campus. The institute was founded in 1970 through the initiative of the Norwegian and Swedish Save the Children Organizations. AHRI has got its name from the Norwegian physician, Gerhard Henrik Armauer Hansen, who first described the leprosy bacillus (*Mycobacterium leprae*) as a causative agent of leprosy. AHRI conducts basic and applied research in the field of leprosy, tuberculosis, leishmaniasis, HIV, malaria and other infectious diseases. Recently, the portfolio of the institute has expanded to include non-infectious diseases such as diabetes and cancer. FACSCanto II, Luminex, qPCR machine, ELISA and ELISPOT readers, BACTEC and HLA typing instruments are among the main core facilities available at AHRI.

5.1.3. The study design

A case-control study design with follow-up was chosen for this study. A case-control study is a type of observational study which involves two groups, cases and controls. Cases are a group having the desired outcome and controls are a group sharing all properties with cases except the desired outcome in a given population. The cases and controls should be drawn from the same population.

In contrast to a cohort study, in a case-control study, we start with well-defined cases having the desired outcome variable and another group called controls from the same population but doesn't have the desired outcome. In a cohort study, we begin with one group free of the desired outcome and prospectively classify them according to their exposure to the putative risk factor. A case-control study is one of the most frequently used study designs to investigate the aetiology of diseases. A case-control study is often used to identify factors that may contribute to a medical condition by comparing subjects who have the condition "the cases" with patients who do not have the condition "the controls". It is particularly suitable for the study of diseases having long incubation period like leprosy. This is because in a case-control study we start with

study subjects who have already developed the condition of interest. The desired outcome of this study was a patient with lepromatous leprosy who has ENL reactions. Hence, waiting a long time for the condition of interest to occur was not required as in a prospective cohort study. Furthermore, a case-control study, is cost-effective and one of the best study designs for examining multiple exposures without compromising the significance of the result. However, in a case-control study it is very difficult to determine the temporal sequence between exposure and outcome and hence usually the odds of occurrence is reported but not the odds of incidence. To avoid selection and recall bias in a case -control studies, the use of incident cases than prevalent cases is advisable. Incident cases are newly diagnosed cases with the desired interest (desired outcome) during a defined time period while prevalent cases are cases who have had the outcome under investigation for some time. The temporal sequence of exposure and disease is easier to assess among incident cases than among prevalent cases.

In this study, a nested case-control study was adopted to e ensure that cases (patients with ENL reactions) and controls (LL patients without ENL reactions) at the time of diagnosis were drawn from the same study population. A three-time case -control study design was used to measure the immunological status of cases and controls during the course of treatment for the desired outcome (leprosy and leprosy reaction). A total of 77 patients with leprosy, of which 46 were patients with ENL and 31 non-reactional patients with LL were cases and controls respectively. Individuals in each groups were followed for 28 weeks. Clinical and laboratory examination were done at each visit. Blood samples were obtained three time from each patient, while biopsies were obtained twice (at recruitment and after treatment) from each patient.

We applied a proper and restrictive case definition, well-defined controls, pre-set hypothesis, a pre-defined outcome variable, clearly established inclusion and exclusion criteria and adequate sample size to arrive at a valid conclusion.

5.1.4. Source, study and sample population

The source population is the population from which the study subjects are drawn. It is the catchment population. It refers to the totalities of those that share, at least, one common factor. In our study, the source population were all patients with leprosy visiting ALERT hospital during the study period. The common property of the

population was that all of them had diagnosed leprosy, although their clinical forms of leprosy in the population varied.

The study population is the population from which the samples are drawn. It is the population to which the results of the study may be extrapolated out to. However, the extrapolation depends on the design and objective of the study. Study population sometimes referred to as target population. The study population are more uniform than the source population. Our definition of the study population was all patients with ENL and LL attending Red Medical Clinic at ALERT hospital during the study period.

The sample population is the population which comprises of individuals drawn from the study population. Study population and sample population share identical properties of a subject of interest for the researcher. For example, in our ENL study, all patients with ENL cases were new, untreated with corticosteroids or previously treated but off corticosteroids at least for 15 days. Hence, all these patients with ENL visited RMC during the study period are study population. For many reasons, it is not possible to study all untreated patients with ENL which necessitates the selection of some patients with ENL from the group using either probability or non-probability sampling methods. Due to the very close nature of study population and sample population, most researchers use sample and study population interchangeably. The following diagram shows the difference among the three populations schematically (Figure 5.1).

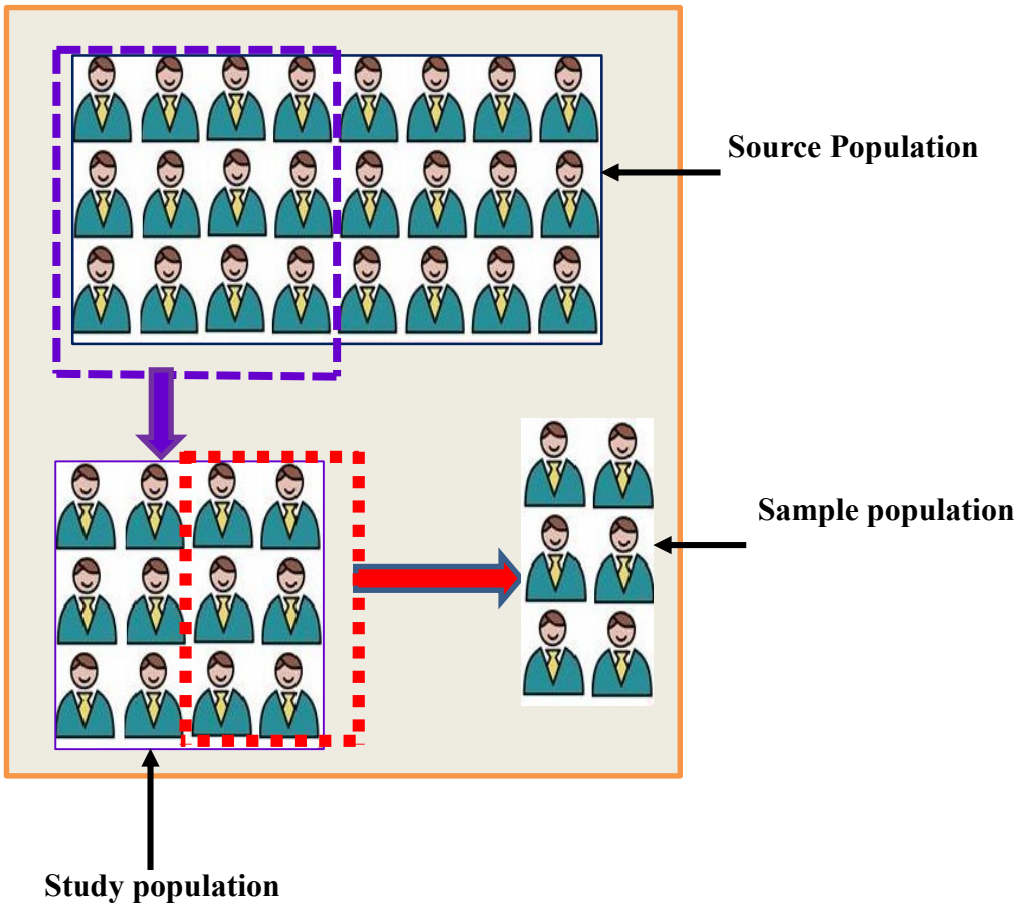


Figure 5.1. Schematic representation of source, study and sample population

The following definitions were used for source, study and sample population in our ENL study.

Source population: All patients with leprosy who presented at the leprosy Clinic (RMC) at ALERT Hospital during the study period.

Study Population: Included all patients with ENL reactions and non-reactional LL patients.

Sample population: All selected and consenting patients who were eligible for the study.

5.1.5. Sample size determination

Sample size determination is the process of choosing the number of observations or replicates required to include to a statistical sample. The sample size is a vital feature of any study if drawing inferences about a population from which the samples drawn is required. Determining the ideal sample size for a study guarantees an adequate power to detect statistical significance. Hence, it is a critical step in the design of a planned clinical studies (Suresh and Chandrashekara, 2012). Power is the probability of correctly rejecting the null hypothesis. It is possible to increase power by increasing the sample size but as sample size becomes too large, an effect of little scientific importance is statistically detectable and leads to a significant difference between groups. Considering an appropriate sample size is also important for economic reasons. Hence, power and sample size determination are a fundamental step in the design of clinical research. A good clinical study design is judged in terms of the estimate of the effect size, confidence interval and P-value. The confidence interval is used to indicate the likely range of values for the given effect size in the population and P-value explains how likely that the observed effect in the sample is due to chance. Although sample size determination is theoretically simple, practically it is one of the challenges in a clinical study, particularly in immunology. Immunological studies are very expensive and time-consuming. Therefore, it is mandatory to balance the sample size and the economics.

In our study, we calculated the sample size assuming a constant probability of exposure throughout the pool of controls. The controls were matched to cases in terms of age, gender or duration of the onset of leprosy. To calculate the sample size for each group (cases and controls), the percentage of FoxP3 expression on $CD4^+CD25^+$ was considered. The percentage of FoxP3 expression in $CD4^+CD25^+$ T-cells was used for sample size calculation since the main goal of the study was describing the relative percentage of regulatory T-cells in ENL and LL controls. As mentioned in the preceding sections, FoxP3 is the robust Treg marker reported by several studies. A 95% confidence interval with 5% level of significance and 80% power was used to obtain reasonable sample size. The expected percentage of Tregs ($CD4^+CD25^+$ $FoxP3^+$) in patients with ENL and LL controls was 1.2% and 2.8% respectively (Attia et al., 2010). Based on this, using STATA 12 the required sample size for each group was calculated to be 30. Anticipating 50% defaulter rate in patients with ENL cases,

the total sample size required for this study was adjusted to 75 (45 ENL cases and 30 LL controls). Group one: 45 patients presenting with ENL (cases), Group two: 30 non-reactional LL patients (controls)

5.1.6. Effect size

Using the G*power 3.1.7, software, the effect size was calculated as; Input parameters: α=0.05, 1-β=0.8., N=75, test criteria: one tail and effect direction: H1 > Ho. The calculated effect size (Se) is 0.198. Interpretation: when the value of the standard error of the mean for the control and experimental group is $\geq$ 0.198, there is a significant difference between the two groups with an effect size of 0.198 (Figure 5.2).

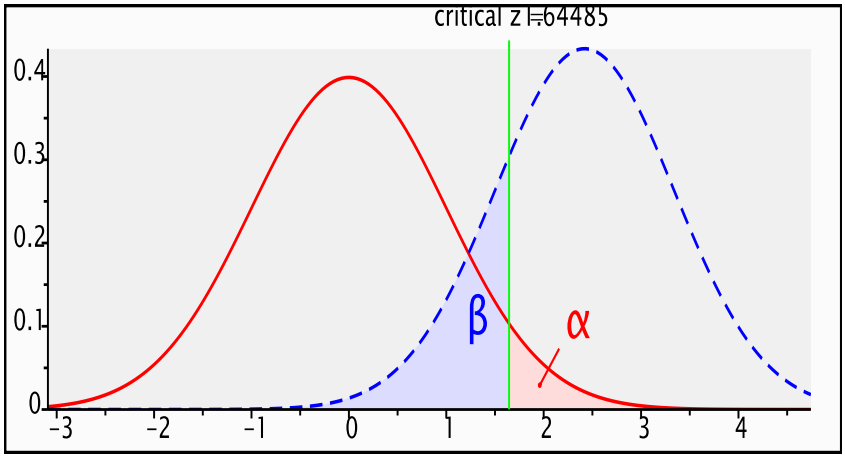


Figure 5.2. : Standardized normal distribution for Z score showing critical regions for the desired effect size.

5.1.7. Inclusion and exclusion criteria

Inclusion criteria

Patients with leprosy who fulfilled the following criteria were eligible to participate in the study and considered members of the sample population.

i. Age group: 18-65 years old
ii. Individuals who gave consent and were willing to return for follow-up study
iii. Individuals diagnosed as having leprosy with clinical evidence of ENL reaction or

iv. Individuals diagnosed as having leprosy with clinical evidence of non-reactional LL and matched with one of the cases at least by age or sex.

Exclusion criteria

Any individual characterized by at least one of the following criteria was not eligible to participate in the study.

i. Individuals unwilling to give consent or unwilling to participate in the follow-up study
ii. Leprosy patients with other forms of the clinical spectrum of leprosy (TT, BT, BB, BL).
iii. Children below 18 and adults above 65 years old.
iv. Individuals whose weight was below 40kg.
v. Pregnant and lactating mothers
vi. Anaemic patients
vii. Patients with a history of concomitant severe conditions such as TB, HIV/AIDS, cardiac and renal problems, diabetes mellitus (DM) that potentially might affect the evaluation of the patient's immune profile.

viii. ENL patients taking prednisolone or completed prednisolone treatment 14 days or less.

5.1.8. Ethical considerations

Since our study involves human subjects and human samples, we adhered to the ethical principles and code of practice recommended by the World Medical Association declaration of Helsinki 1964 and later revisions. Training of transferable skills training offered by LSHTM (research ethics) and good laboratory practice (GLP) offered by Armauer Hansen Research Institute were obtained prior to the commencement of the study. Research ethical approvals were obtained from London School of Hygiene and Tropical Medicine Ethics Committee, AHRI/ALERT Ethics Review Committee (AAERC) and National Research Ethics Review Committee (NRERC), Ethiopia. Support letters were obtained from ALERT Hospital, AHRI, and LSHTM.

5.1.9. Informing and consenting patients

Information sheet and consent were prepared in English and translated to Amharic (local language) by a native speaker (Appendix 1). The information sheet was written in plain words and given to a layperson to verify if the information was easily understandable by the participants. The information sheet was given to each participant to read or was read for the participant. Then, the participant was requested to notify his/her decision.

We recruited a study coordinator with a public health professional who has been trained for research ethics and consenting patients and does not involve in leprosy diagnosis and treatment to avoid any discomfort or worries anticipated by patients. Candidate participants were informed that they had the right to refuse to participate without given reasons. They were also assured that they could withdraw at any time from the study without giving reasons and without prejudicing further treatment. Participants who were fulfilled the initial assessment were invited to take part in the study. For consenting the two main local languages, Afan Oromo and Amharic were used for the majority of the participants. For some patients who were unable to speak these two languages, a translator was used. Consent was obtained from each study subject by the study coordinator. If either the treating physician or nurse takes consent, patients could give consent irrespective of their voluntariness because they may think that it could affect the outcome of their treatment.

In Ethiopia donating blood is not encouraged by many societies, probably tied to religion or cultural aspects. The challenge is more serious when more volume of blood is required (more than 10mL). Hence, we approached the participants carefully and explained them the normal volume of blood donated by apparently healthy adults such as for blood bank to convince them to donate 40-50mL blood.

The consent form was signed by all participants before inclusion to the study. For participants unable to sign, a thumb print was obtained instead and witnessed by a person obtaining the consent. A study code was given for each participant and on the participant's consent form only the code was written to maintain confidentiality. The study code and the name of the participant were written on the separate log book and accessed only by the principal investigator and the clinical coordinator.

5.1.10. Study subjects recruitment

Study subjects were recruited at Red Medical Clinic of ALERT Hospital which is dedicated to leprosy diagnosis, treatment, and rehabilitation. There are 5 nurses of which 2 are leprosy specialist nurses. There are also 5 dermatologists (2 females and 3 males) engaged in leprosy diagnosis and treatment. Theoretically, one dermatologist is assigned every day to see leprosy patients at the Clinic. However, practically almost all patients are seen by nurses.

At ALERT Hospital, suspected leprosy cases are sent to Red Medical Clinic from central triage or from the department of dermatology after they are seen by a dermatologist for further diagnosis and treatment. I had a brief discussion with all nurses working in the clinic about the plan and the requirements of the study. There were many challenges to reach a consensus. Low staff morale, shortage of medication, incentive and negligence were some among the challenges. We agreed with the nurses to direct new and untreated patients with ENL cases to the study coordinator for consenting. The study coordinator has been working in occupational therapy department. Participants were guided by the assigned porter to donate a sample, collect their medication, receive compensation and given the appointment for the second and third round visits. Following consenting, clinical data were filled on the pre-structured questionnaire at RMC and checked by the coordinator for completeness. Bacillary index (BI) and other laboratory examination were done for further confirmation. Then patients were guided to the AHRI blood and biopsy sample room to donate blood and

skin biopsy sample. After sample collection, each participant was compensated for transportation and lunch. The next appointment date was given in written form on the specifically designed participation ID card (Appendix 1).

5.1.11. Sampling procedure

During the study period, 46 patients with ENL and 31 non-reactional LL controls were recruited as case and control study subjects respectively. Any measurement and assessment information obtained from each group at the beginning was used as a baseline data. Samples from each patient at his/her next episode of ENL were collected to look at the consistency of immunological events within the patient.

Samples were collected at start, 12 and 24 weeks after the start of corticosteroid administration. The 12th week was chosen since the steady decrease of steroid after12th week reaches less than half of the start dose and after 24th-week steroid administration normally off unless the patient experiences a chronic condition (Figure 5.3). The samples obtained at recruitment were used to analyse the immunological profiles (Regulatory and memory T-cells, pro-inflammatory cytokines and regulatory cytokines) before treatment. The second time point samples were obtained when ENL cases completed half of their corticosteroid treatment time and the samples were used to determine these immune profiles while the patient was on the corticosteroid treatment. The third time point sample was obtained when an ENL patient completed corticosteroid treatment and the free treatment period is 15 days or more. Skin Biopsy samples were obtained at entry and exit only.

5.1.12. Follow-up

A single point of contact was set for patients after enrolment to the program. They were advised to report any changes regarding their disease condition (leprosy) to the person in charge (coordinator). They were also linked to the nearby health stations in their village. The health stations were contacted and notified to send the patient to ALERT hospital in case they develop reactions (non-reactional LL controls) or for any worsening conditions. The second round sample was collected from each patient when the initial prednisolone dose was tapered by half. The third round sample was collected after the patient was off prednisolone for 15 days confirming the patient had no evidence of new ENL lesions. In those patients who did not show improvement,

prednisolone administration continued as prescribed by the physician and samples were collected at the pre-set time interval.

5.1.13. Patient compensation and appointment for next sampling

Each participant was compensated for transportation and lunch at each visit. For those patients coming from the outsides of the outskirts of Addis Ababa, accommodation was provided. The next visiting date was given on the specifically designed ID card. Three days before the appointment each participant was reminded by the phone call. For individuals who did not have their own phone number, we used either relative's phone number if available or the nearby health post's contact number.

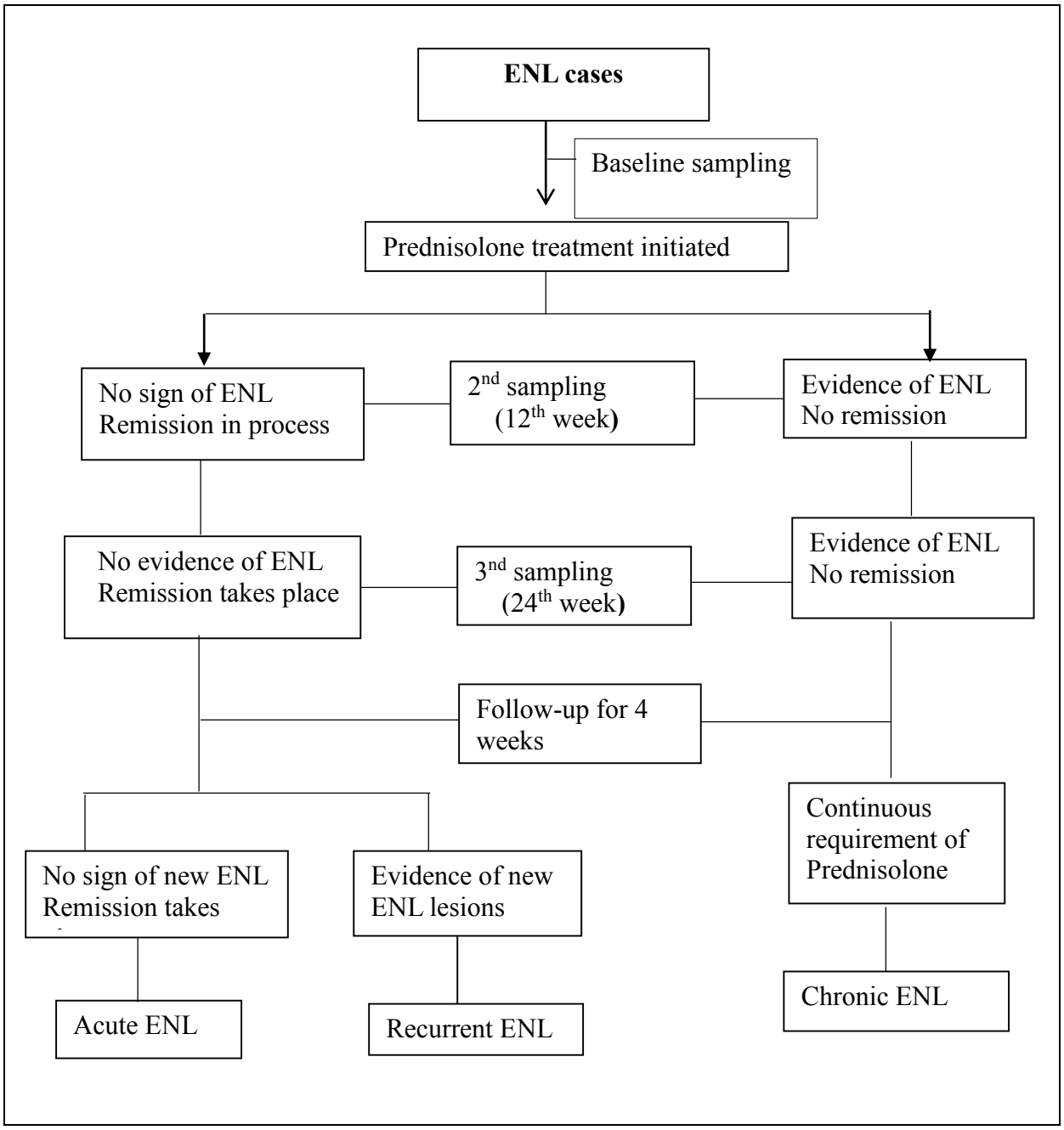


Figure 5.3. ENL cases sampling and follow-up chart

5.1.14. Demographic and clinical data collection

Structured questionnaire were used for clinical data recording for each participant. The ENL International STudy group (ENLIST) format was modified and used for clinical data recording. The data collection sheet included the demographic, clinical and diagnostic information set following the standard guideline. The clinical information included core points such as the clinical feature, skin lesion, nerve functions and systemic involvement. Clinical assessment was done three times consecutively. At recruitment, the clinical assessment included the socio-demographic and details of clinical signs and symptoms and used as a baseline information. During the second and third-time visit assessment, socio-demographic data were not recorded to avoid unnecessary duplication.

The baseline data included card number, study code number, age, sex, weight, blood pressure, time since leprosy symptoms developed, the Ridley-Jopling classification of the disease, previous treatment with MDT and reactions. A detailed history of the skin examination and nerve symptoms were recorded on the form. The number, size and morphology of skin lesions, the presence of erythema or ulceration, the presence of peripheral oedema, nerve tenderness, nerve pain or paraesthesia were recorded on the clinical data recording form. The physiotherapist performed Sensory Testing (ST) using semmes-weinnstein monofilaments (SWMs) at designated test sites on the hands and feet and Voluntary Muscle Testing (VMT) result was documented for each participant during each visit. For all participants, basic laboratory tests were obtained. These tests included full blood count, haemoglobin concentration, renal function, liver function, glucose level and stool specimen for the presence of ova, cysts and parasites. Symptomatic screening for TB (a cough longer than 2 weeks, night sweats, weight loss) and HIV test were performed for each participant at each visit. Slit-skin smear was used from four sites to calculate the BI at the time of enrolment. BI at leprosy diagnosis and the current BI were recorded on the form for further analysis.

SECTION 2: CLINICAL SAMPLE COLLECTION AND PROCESSING

Blood and skin punch biopsy samples were obtained from all patients with ENL and LL patient controls. Forty to forty-five- millilitre blood was collected in vacutainer test tubes for the subsequent use in flow cytometry and ELISA. About 2mL of blood was collected in PAXgene Blood RNA Tubes for mRNA isolation. Blood samples were obtained before, during and after treatment of ENL cases and LL controls. Biopsy samples were collected in three different preservatives: for histopathology in 10% formalin, for mRNA isolation in 1mL RNAlater and for immunohistochemistry embedded in optimum cutting temperature (OCT) medium. Procedures for each sample collection and storage are described below.

5.2.1. Clinical sample collection

Blood samples were collected from each patient at each visit. Skin punch biopsies were taken at entry and exit only. Blood sample (40-45mL) was collected in sterile BD heparinised vacutainer ® tubes (BD, Franklin, Lakes, NJ, USA) and 2mL of blood was added into PAXgene® Blood RNA Tubes (PreAnalytix, GmbH, Switzerland) following the aseptic technique. The phlebotomists in charge (the nurses) were documented the date, study code number, ALERT number, AHRI number, sex, age, blood volume, body sites from which biopsy was taken, name and signatures of the nurse in charge. The nurses were also instructed to make sure that the patient is the correct participant from whom blood and biopsy sample is desired by crosschecking with the attached document. Two research nurses who had been trained and have experience in leprosy diagnosis and treatment were recruited for drawing blood and to take punch biopsy samples.

A patient was lied down on its back on the bed while drawing blood and taking biopsy samples. The heparinised vacutainer was labelled with patient study code and date. For skin punch biopsy, three types of preservation media were prepared as described before. One 4mm punch biopsy was fixed in 10% formalin and kept at ambient temperature to be used for histology. Another 4mm punch biopsy taken from the same area was transferred to a Nunc® tube containing 1mL RNAlater® solution (Thermo-Fisher Scientific) and was kept at -20^{o}C for 48 hours and then transferred to -80 ^{o}C freezer. The third 4mm punch biopsy sample was placed in tissue-Tek cryomold and embedded by optimum cutting temperature compound (Tissue-Tek®, O.C.T.

compound, Sakura® Finetek, USA) and then snap-frozen in liquid nitrogen. The Frozen biopsy then transferred to -80^{o}C for storage until used for immunohistochemistry. The PAXgene Blood RNA Tubes were kept at room temperature for 2 hours and then transferred to -80^{o}C for storage until used for RNA isolation. Blood in heparinised vacutainer tubes was brought to PBMCs isolation room and kept for 1 hour at room temperature in disinfected sterile safety cabinet and then followed by PBMCs isolation.

5.2.2. Peripheral blood mononuclear cells isolation

Peripheral blood mononuclear cells (PBMCs) are blood cells such as monocytes and lymphocytes with a round shaped nucleus as opposed to granulocytes which have lobbed nucleus. The lymphocytes population consist of T- cells, B- cells and natural killer (NK) cells. Lymphocytes are the main component of the immune system, playing an essential role in the host's defence system. The separation of PBMCs from whole blood is usually done through density gradient using Ficoll. After the centrifugation step, the Ficoll separates blood into layers of plasma, PBMCs, granulocytes and red blood cells (RBCs) (Figure 5.4).

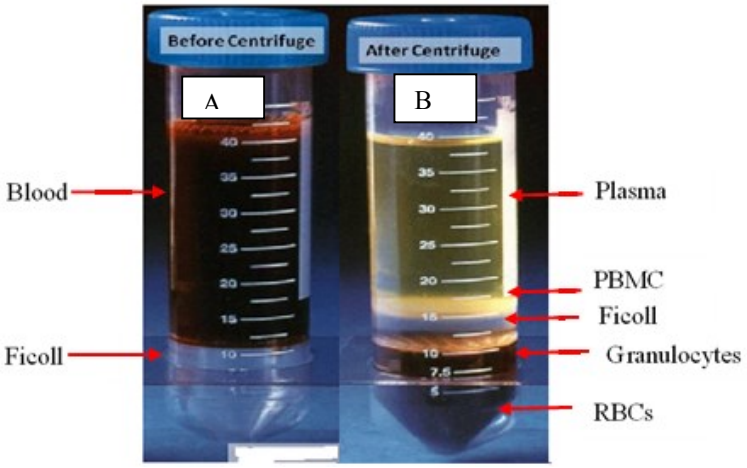


Figure 5.4. PBMCs isolation from whole blood A) Before centrifugation B) After centrifugation with Ficoll-paque.

PBMCs isolation from whole blood is the critical step for the subsequent works. In our study, PBMCs isolation was optimized using healthy volunteer blood. The PBMCs isolation protocol is briefly described as follows: Blood samples in heparinised tubes brought to safety cabinet and kept for one hour at room temperature (RT). Fifteen millilitres of Ficoll-paque™ PLUS (GE Healthcare, UK LTD) kept at RT was added to Leucosep™ tubes (Greiner Bio-one, USA) and centrifuged at 1000g for 30 seconds to make the ficoll-paque go down beneath the porous barrier (Figure 5.4A). After inverting the blood containing tube 2 to 3 times, 25mL of blood from the heparinised tube was poured slowly to the Leucosep tube and centrifuged at 800g for 25 minutes with break off at RT. After the spinning, the plasma (top layer, Figure 6.4) was transferred to cryotubes and the PBMC interface was transferred to 50mL of non-pyrogenic, and RNase/DNase-free Corning® (BD, UK) centrifuge tube containing 15 mL of pre-warmed to RT buffered phosphate saline solution (PBS, Sigma Aldrich®, UK). Then the tube was filled with PBS and centrifuged at 800g for 10 minutes at RT with a break on low. After the spinning, the supernatant was discarded and the pellet was resuspended with 1mL of Roswell Park Memorial Institute (RMMI medium 1640 (1x) + GlutaMAX™ + Pen-Strip (GBICO™, Life technologies™, UK) and mixed thoroughly and then the volume brought to 10 mL by adding complete RPMI medium followed by washing at 600g for 10 minutes at RT with break on low. After this wash, the supernatant was discarded and the pellet was resuspended by adding 1mL of complete RPMI medium and mixed thoroughly until the pellet dissolve and then the volume was adjusted to 2mL. Ten µl of the sample was mixed with 90µl of 0.4%, sterile, filtered Trypan Blue solution (Sigma Aldrich®, UK) to count live cells. Ten µl of the mix was mounted on Neubauer Chamber (Figure 6.5). Cells in the 25 inner squares of Neubauer Chamber were counted and the total number of cells/mL was obtained by multiplying the counted cell number by the dilution factor (10x), the volume of the cell (2mL) and the constant (10^4). For example, if 200 cells counted in the inner 25 squares of the chamber, the total number of cells in the sample will be: $200 \times 10 \times 2 \times 10^4 = 40 \times 10^6$ cells/mL (Figure 5.5).

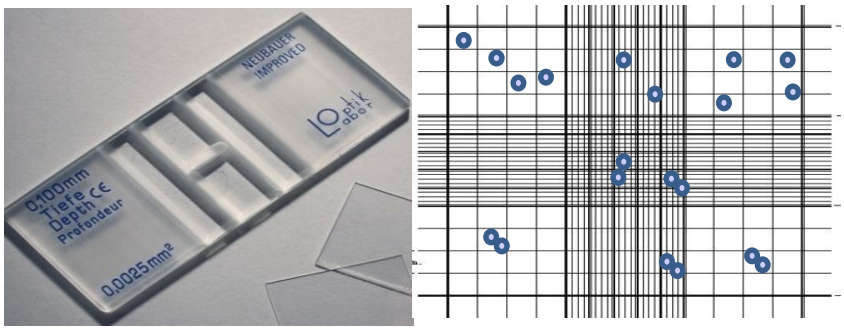


Figure 5.5. Cell counting chamber. A) Neubauer Chamber B) counting squares

5.2.3. Freezing cells

Cells resuspended in a half the final volume of media with 20% Foetal Bovine Serum (FBS, heat inactivated, endotoxin tested ≤5 EU/ml, (GIBCO® Life technologies, UK) and kept at 4^0C. The freezing medium was prepared with pre-cooled RMMI medium 1640 (1x) + GlutaMAX™ + Pen-Strip containing 20% foetal bovine serum and 20% Dimethyl sulfoxide (DMSO) molecular biology grade (Sigma Aldrich®, UK). Then the freezing medium was gently pipetted to the sample and 1mL of the aliquot containing 5-10 million cells was pitted to the 2mL volume cryovials quickly. The cryovials were transferred to a pre-cooled MRFrosty containing isopropanol (Figure 5.6). Then, the MRFrosty was kept at -80ºC for freezing the cells slowly. After 24 hours, the cryovials were transferred to cryobox and stored at -80ºC until used.

Figure 5.6. The freezing can (MRFrosty)

5.2.4. Procedures for thawing frozen PBMCs

Thawing frozen PBMCs is a critical step for subsequent process. Frozen cells are fragile and should be handled gently with maximum care. If PBMCs are not thawed properly, viability and cells recovery can be compromised. We optimized the procedure for thawing cells and used the optimized protocol for thawing frozen PBMCs as described by Thompson et al. (2001). The procedure for thawing frozen PBMCs is briefly described as follow as: Thawing media (complete media + 0.01% Benzonase) was prepared and incubated in a 37^{o}C water bath. The cryovials from liquid nitrogen or -80 ^{o}C were transferred to a 37^{o}C water bath and kept 1 to 2 minutes until 75% of the cells thawed. Then the surface of cryovials were cleaned with 70% alcohol and the cells were transferred to 15mL polypropylene centrifuge tubes containing 10mL of pre-warmed thawing media. Then the cells were centrifuged at 600g for 7 minutes. The supernatant was decanted and the tube was flicked by a finger to break the pellet. The pellet was resuspended with 5mL complete media and centrifuged at 600g for 5 minutes at RT. After decanting the supernatant, the pellet was dissolved in 1mL completed media. Then cells were counted with a haematocytometre (Neubauer chamber) and the cell viability was determined with trypan blue as previously described. Cell concentration was adjusted to 10^{6}cells/mL. Finally, 1mL/well cell suspension was plated on 24 well polystyrene cell culture plate (Corning® Costar® cell culture plates) and incubated at 37^{o}C in a 5% carbon dioxide incubator overnight. After overnight incubation, cells were brought to flow cytometry staining room for staining with fluorochromes conjugated antibodies (described under flow cytometry topic, section 3).

SECTION 3: FLOW CYTOMETRY

5.3.1. Flow cytometry and Fluorochromes

Flow cytometry: Flow cytometry is a laser-based technique that simultaneously measures and then analysis multiple physical characteristics of single cells as they flow in a fluid stream through a beam of light. The properties measured include the relative size of cells, relative granularity or internal complexity and relative fluorescence intensity. Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry which provides methods for sorting for mixtures of biological cells into groups based upon the specific light scattering and fluorescent characteristics of each cell. FACS is a trademark and owned by Becton, Dickinson Company.

Fluorochromes used in flow cytometry: A fluorochrome is a chemical that can re-emit light upon light excitation. Each fluorochrome has specific absorption spectrum, extinction coefficient, emission spectrum and quantum efficiency. These fluorochromes are conjugated to antibodies for use in flow cytometry. Fluorochromes are emitted at blue, green, red or violet laser of the flow cytometer. For example Fluorescein Isothiocyanate (FITC), PE-Cyanine 5.5 and PE-Cyanine 7 are among fluorochromes which have 488nm excitation and emitted at blue laser while APC-eFluor® (633) 780 fluorochromes are used for the red laser of the flow cytometer.

5.3.2. Panel construction

Antibody panel design for multicolour flow cytometry involves the process of choosing fluorochromes from each channel and setting a panel. The panel construction depends on the question to be answered by the panel, knowledge of the flow cytometer, antigens, fluorochrome intensity (brightness) and other factors. It also involves the choice of the controls to be used. Such controls include compensation control, isotype control, Fluorescence Minus One (FMO) controls, unstained controls and gating controls. FMO controls contain all the fluorochrome markers except the one of interest. For low density or smeared population such as regulatory T-cells, FMO allow accurate delimitation of positively versus negatively stained cells.

5.3.2.1. Regulatory T cell panel

To describe Tregs we used 8 markers. The markers used for this panel included anti-human CD3 (APC 450), anti-human CD4 (eFluoro780), anti-human CD8 (PerCp-Cy5.5), anti-human CD25 (PE-Cy7), anti-human CD127 (APC), anti-human CD161 (PE), anti-human FoxP3 (FITC) and a viability stain (Aqua, v500). We used FMO for each maker, compensation controls, unstained and Isotype controls whenever necessary (Table 5.1). Cell viability was also checked before the staining using 0.4% trypan blue.

5.3.2.2. Memory T-cell panel

Six markers were used to determine memory and activated T- cells. These markers were: anti-human CD3 (APC 450), anti-human CD4 (eFluoro780), anti-human CD8 (PerCp-Cy5.5), anti-human CD62L (APC), anti-human CD45RO (PE), and viability (Aqua, v500) (Table 5.1). We used for each maker FMO, compensation controls and unstained cells. Unstained cells were used to exclude the autofluorescence of cells. Cell viability was also checked before staining using 0.4% trypan blue.

5.3.2.3. B- cell Panel

To describe the memory B-cells sub types the following markers were used: anti-CD10 (FITC), anti-PD1 (PE), anti-CD19 (PerCp-Cy5.5), anti-CD95 (PE-Cy7), anti-CD27 (V500), Anti-CD21 (V450), Live/dead (eFluoro 780), and Isotype control (IgG1) (Table 5.1). Compensation controls and unstained cells were used as described before. Cell viability was also checked before staining using 0.4% trypan blue.

Table 5.1. Antibody panels, fluorochromes and sources of antibodies used in the study

Panel name	Antibody	Conjugated fluorochrome	Clone	Source	Reactivity
Regulatory T -cells	anti- CD3	APC 450	UCHT1	eBioscience	Human
	anti- CD4	eFluoro780	OKT4	eBioscience	Human
	anti-CD8	PerCp-Cy5.5	RPA-T8	eBioscience	Human
	anti-CD25	PE-Cy7	M-A251	BD	Human
	anti-CD127	APC	eBioRDR5	eBioscience	Human
	anti-CD161	PE	HP-3G10	eBioscience	Human
	anti-FoxP3	FITC	PCH101	eBioscience	Human
	Live/dead stain	V500 aqua	-	Life technologies	-
Memory T- cells	anti -CD3	APC 450	UCHT1	eBioscience	Human
	anti-CD4	eFluoro780	OKT4	eBioscience	Human
	anti-CD8	PerCp-Cy5.5	RPA-T8	eBioscience	Human
	anti-CD45RO	PE	UCHL1	BD	Human
	anti-CD62L	APC	DREG56	BD	Human
	Live/dead stain	V500 aqua	-	Life technologies	Human
B- cells	anti-CD10	FITC	W8E7	BD	Human
	anti-PD1 (CD279)	PE	EH12.1	BD	Human
	anti-CD19	PerCp-Cy5.5	HIB19	BD	Human
	anti-CD95	PE-Cy7	DX2	BD	Human
	anti-CD27	V500	M-T271	BD	Human
	Anti-CD21	V450	B-ly4	BD	Human
	Live/dead	eFluoro 780	-	Life technologies	-

5.3.3. Antibody titration

In flow cytometry, antibody titration is a process of determining the right concentration of antibody to obtain an optimum signal. This is related to antibody-antigen kinetics since the goal in flow cytometry is saturation of the marker with the antibody. Hence, the purpose of titration is to obtain the optimum concentration where binding saturation occurs. Low antibody concentration results in dim staining while the use of a high antibody concentration will increase false positivity by staining the background. The optimal antibody titre is the dilution that produces the maximum signal to noise ratio and it is often not the concentration that produces the highest percentage of positive cells (Stewart and Stewart, 1997).

To determine the optimum antibody titres required to stain of the samples (PBMCs), each antibody was titrated in four dilutions by taking the company's recommendation as the top dilution. Cells were used for titrating antibodies. Unstained cells were used as a negative control. A single staining protocol that means each antibody was stained individually. The median fluorescent intensity of positive (MFIpos) and negative population (MFIneg) was then calculated. The standard deviation for the negative population (SDneg) was also obtained. Then the staining index, named as a signal to noise ratio (S/N) was calculated for each antibody and a scatter plot was obtained for the log antibody concentration versus the staining index. As a rule, the higher the S/N, the better is the signal (Maecker and Trotter, 2004). In the following example, (Figure 5.7) 2.5µl of antibody is the optimal volume to be used for the 100µl final volume of staining.

$$S/N=(MFIpos - MFIneg)/2SDneg$$

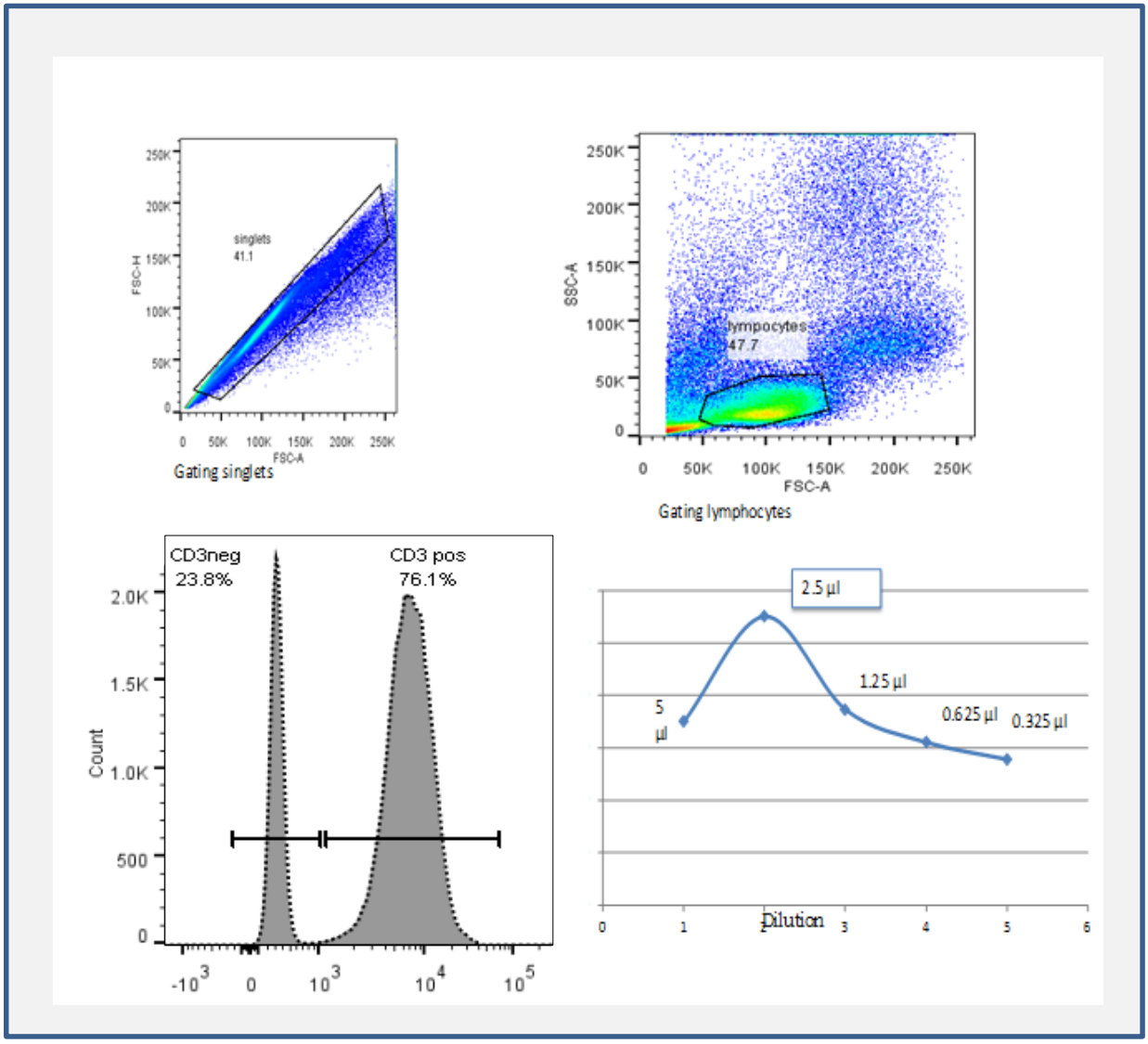


Figure 5.7. Antibody titration: determination of the optimum amount of antibody required for single stain by graphical methods.

5.3.4. Nuclear staining (staining for Tregs)

The staining of Tregs involves two steps: surface staining followed by nuclear staining. The nuclear staining involves permeabilization of the cells to facilitate the entrance of fluorochromes-conjugated antibody into the nuclear region to stain the marker of interest which is FoxP3 in this study. The staining procedure is briefly described in the following paragraphs.

Frozen PBMCs were thawed as described previously. Cells were washed twice with RMPI-1640 supplemented with benzonase (10 U/ml, Novagen, Merck4Biosciences, Merck KGaA, Darmstadt, Germany). After washing, the pellets were resuspended in 1mL complete media.

About $1x10^6$/mL cells were plated on 48 well cell culture plate with plain media (without stimulation) overnight for resting cells. Then the cell suspension was transferred to round bottomed FACS tubes (Falcon®, BD, UK) followed by spinning at 400g for 5 minutes at RT. After discarding the supernatant, the pellet was resuspended in 1mL phosphate buffered saline solution (PBS, Sigma Aldrich®, UK) and centrifuged at 400g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 50µl of PBS. Then the cells were incubated in 1mL of 10% human AB serum (Sigma Aldrich®, UK) for 10 minutes in the dark at room temperature to block nonspecific Fc-mediated interactions and followed by centrifugation at 400g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 50µL PBS buffer. Life/dead staining was performed at a concentration of 1µl /1mL live/dead stain (V500 Aqua, Invitrogen, Life technologies, UK) for 15 minutes at 4ºC in the dark. Cells were washed once and stained for surface markers directed against CD3- Pacific blue, CD4- eFluoro 780, CD8- PerCp-Cy5.5, CD127-APC, CD161-PE (all eBioscience, UK) and CD25-PE-Cy7 (BD, Biosciences, UK). The cells were incubated with the cocktail for 30 minutes at 4ºC. After 30 minutes of incubation, the cells were washed with FACS buffer (PBS, 1% FBS, 0.1% NaN3 sodium azide) and followed by intracellular staining. One millilitre of 1xFoxP3 Fixation-Permeabilization buffer (eBioscience, UK) was added to each tube, mixed thoroughly and incubated at 4ºC for 60 minutes. After 60 minutes incubation, without washing 2mL of permeabilization buffer was added and centrifuged at 400g for 5 minutes. The supernatant was discarded and resuspended in 50µl of buffer followed by staining the

permeablized cells with anti-human FoxP3 (FITC, eBioscience) for 30 minutes at 4ºC. Then cells were washed twice by 2mL of permeabilization buffer. Finally, the cells were re-suspended in 400µl FACS buffer and followed by FACS acquisition.

5.3.5. Staining for memory T-cells

Surface staining was performed for the staining of memory T- cells. About $1x10^6$/ml cells were plated on 48 well cell culture plates overnight as described above. Cells were transferred to round bottomed FACS tubes (Falcon®, BD, UK) and washed by PBS as described above. After blocking the Fc receptor-mediated interaction by 1mL of 10% human AB serum, the cells were stained with live/dead stain (V500 Aqua, Invitrogen, Life technologies, UK) for 15 minutes at 4ºC in the dark and washed once followed by staining with anti-human CD3 (APC 450), anti-human CD4 (eFluoro780), anti-human CD8 (PerCp-Cy5.5), anti-human CD62L (APC) and anti-human CD45RO (PE), and incubated at 4ºC for 30 minutes in the dark. After one wash, cells were fixed in 4% paraformaldehyde (PFA, Sigma Aldrich®, UK) for 15 minutes at room temperature. Finally, cells were washed with FACS buffer twice and re-suspended in 400µl FACS buffer for FACS acquisition.

5.3.6. Staining for B-cells

The surface staining protocol used for B cells was similar to that of the staining of memory T-cells except for the different antibody cocktails used. For B-cell staining the following fluorochromes conjugated antibodies were used: anti-CD10 (FITC), anti-PD1 (PE), anti-CD19 (PerCp-Cy5.5), anti-CD95 (PE-Cy7), anti-CD27 (V500), Anti-CD21 (V450), Live/dead (eFluoro 780), and Isotype control (IgG1).

5.3.7. Data acquisition using FACSCanto II

After staining, cells were acquired by flow cytometry. Cell acquisition by flow cytometry involves several setups. The flow cytometry setup techniques used in this study are briefly described in the following sections.

5.3.7.1. Cytometer setup and tracking system

Flow cytometer setup such as laser alignment, laser time delay and sensitivity was checked periodically. Quality control test (QCT) beads supplied by BD were routinely used to test the cytometer's performance. Robust standard deviation of electronic noise

(rSDEN), relative detection efficiency for each detector (Qr), relative optical background (Br), robust coefficient of variation for each setup bead (rCV) and Linearity maximum channel has been checked and monitored by trained system engineer during data acquisition.

5.3.7.2. PMT voltage setting

The voltages on the photomultipler tubes (PMTs) convert the photons of light emitted by the fluorochromes on the cells and convert them to electrons, which eventually become the voltage current that is digitized and ultimately stored in the FCS file as the data output(Maecker and Trotter, 2006).

BD FACSCanto II was used for this study and data were acquired on BD FACSDiva™ software. BD FACSDiva™ software efficiently controls the setup, acquisition, and analysis of flow cytometry data from the BD FACSCanto II workstation. It enables to preview and record data from multiple samples within an automated acquisition process. An acquisition template, experimental layouts and compensation procedures were created at the time of optimization. Unstained cells were used to set optimal electrical voltage for the forward-scattered light (FSC) and side- scattered light (SSC) for each fluorochromes. The voltages were adjusted in such a way that unstained cells appeared in the first quadrant on the logarithmic scale for each fluorochromes. Special attention was given for fluorochromes with longer wavelength emissions such as APC, APC-Cy7, PE-Cy7 and PerCP-Cy5.5 since most cells emit little auto fluorescence at these wavelengths (Wang et al., 2008). The FSC A, H and FSSC A, H were ticked to facilitate the subsequent gating activity.

5.3.7.3. Setting fluorescence compensation

Fluorescence compensation is the process of excluding the spectral overlap when two or more fluorescents are used in multicolour FACS experiments. Spectral overlap is an inherent condition in most multicolour experiments and the spectral overlap should be excluded to avoid false positivity. For fluorescence compensation either cells or beads can be used. However, compensation beads are preferred rather than cells due to the fact that when the markers are dim and or found only on rare subsets of cells, setting compensation is difficult using cells (Maecker and Trotter, 2006).

A single-stained OneComb eBeads (affymetrix, eBioscience, UK) for all fluorescence compensation except for the live dead stain were used. For the viability dye, cells were used for fluorescence compensation. After acquiring the stained compensation beads, the positive and negative population was gated for each single-stained beads and the spectral overlap was made to be calculated automatically and linked to the sample acquisition worksheet (global worksheet) as previously described by Herzenberg et al. (2006). Compensation controls were used for each batch of samples.

5.3.8. Sample acquisition

After the PMT voltage and compensation controls setting, the worksheet area was switched from the normal worksheet to the global worksheet. For inspection purpose, the plots were produced on worksheet such as FSC-H versus FSC-A (to inspect the singlets), FSC-A versus viability marker (to inspect viable cells), SSC-A versus FSC-A (to inspect populations such as lymphocytes, monocytes, granulocytes etc.). The threshold for FSC was set to 5,000. For each sample, 500,000-1,000,000 cells were acquired (Figure 5.8).

5.3.9. The gating Strategy

The acquired data were analysed with FlowJo version 10 (Tree Star, USA). Before gating of cell population for analysis, the acquired cells were inspected for the quality by looking into cell subpopulations expected in the sample (Figure 6.8). Then, for all panels, singlets were gated based on the forward light scatter (FSC) and side light scatter (SSC) properties. Then, the singlets were gated with viability versus SSC-A. Viable cells have low SSC and high FSC scatter (Maecker and Trotter, 2006)(Figure 5.9).

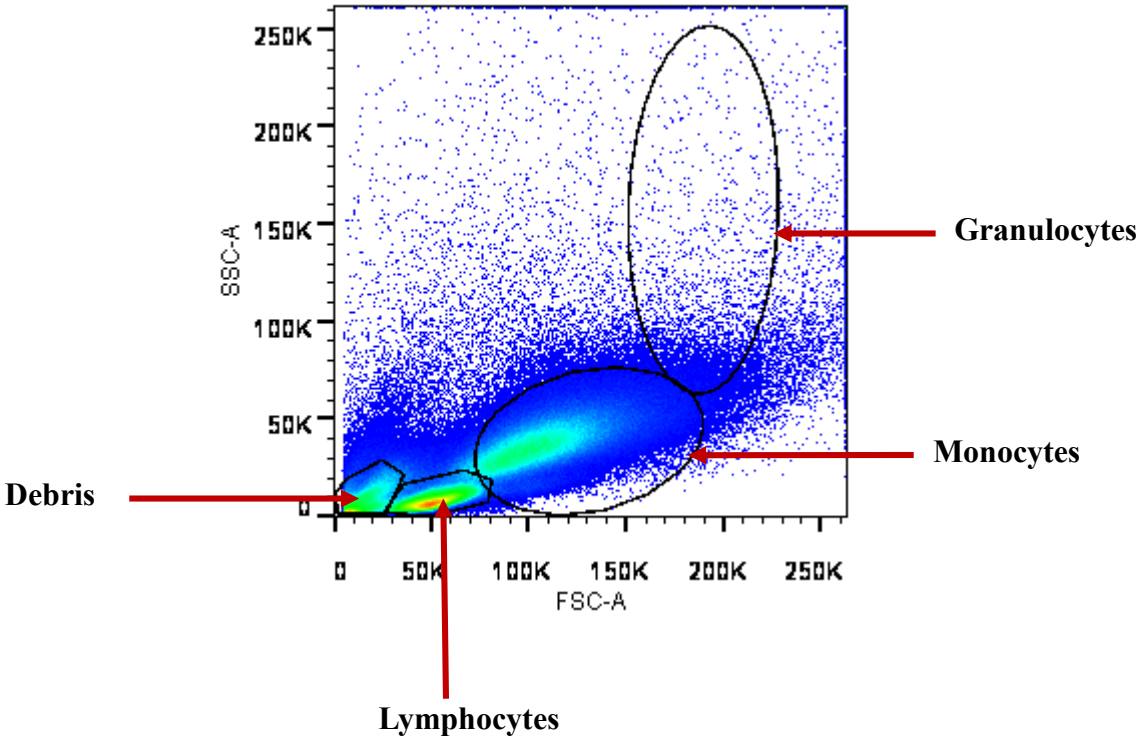


Figure 5.8. SSC-A versus FSC-A of representative frozen PBMCs.

5.3.9.1. Gating Tregs

Viable cells were gated on SSC-A versus FSC-A for lymphocytes. Then, lymphocytes were gated for CD3. Total $CD3^+$ T cells were selected and used for isolation of T-cell subpopulation. $CD3^+$ T cells were gated for $CD4^+$ and $CD8^+$ T cells. The $CD3^+CD4^+$ T cells were used for enumeration of $CD4^+$ T-cell subpopulation. $CD25^+$ cells and $FoxP3^+$ cells were gated on $CD4^+$ T-cells. The double positive cells ($CD25^+FoxP3^+$ cells) were gated with CD127. Then $CD4_+CD25^+FoxP3^+CD127^{lo/-}$ T-cells were considered as $CD4^+$regulatory T-cells. Hence, $CD4^+$ Tregs were defined as $CD3^+CD4^+CD25^+FoxP3^+CD127^{lo/-}$ cells (Shen et al., 2009b).

For $CD8^+$ Tregs similar gating strategy was used and $CD8^+$ Tregs were defined as $CD3^+CD8^+CD25^+FoxP3^+CD127^{lo/-}$ cells. In addition, $CD25^+$ and $FoxP3^+$ cells were also gated on $CD4^+$ and $CD8^+$ cells individually for all samples. CD161 was used as a marker for all IL-17 producing cells. It is previously described that CD161 is a marker for all human IL-17-producing T-cell subsets and is induced by RAR-related orphan receptor C (RORC) (Maggi et al., 2010). However, recent findings have indicated that CD161 can also be expressed on other lymphocytes such as natural killer cells (Fergusson et al., 2014). Therefore, we gated CD161 positive cells on total lymphocytes, T-cells ($CD3^+$ T-cells), $CD4^+$ cells and $CD8^+$ cells individually to catch any IL-17 producing cells. Furthermore, the Boolean gate platform was used to identify all functions within each cell preparation using the full array of possible combinations (Figure 5.9)

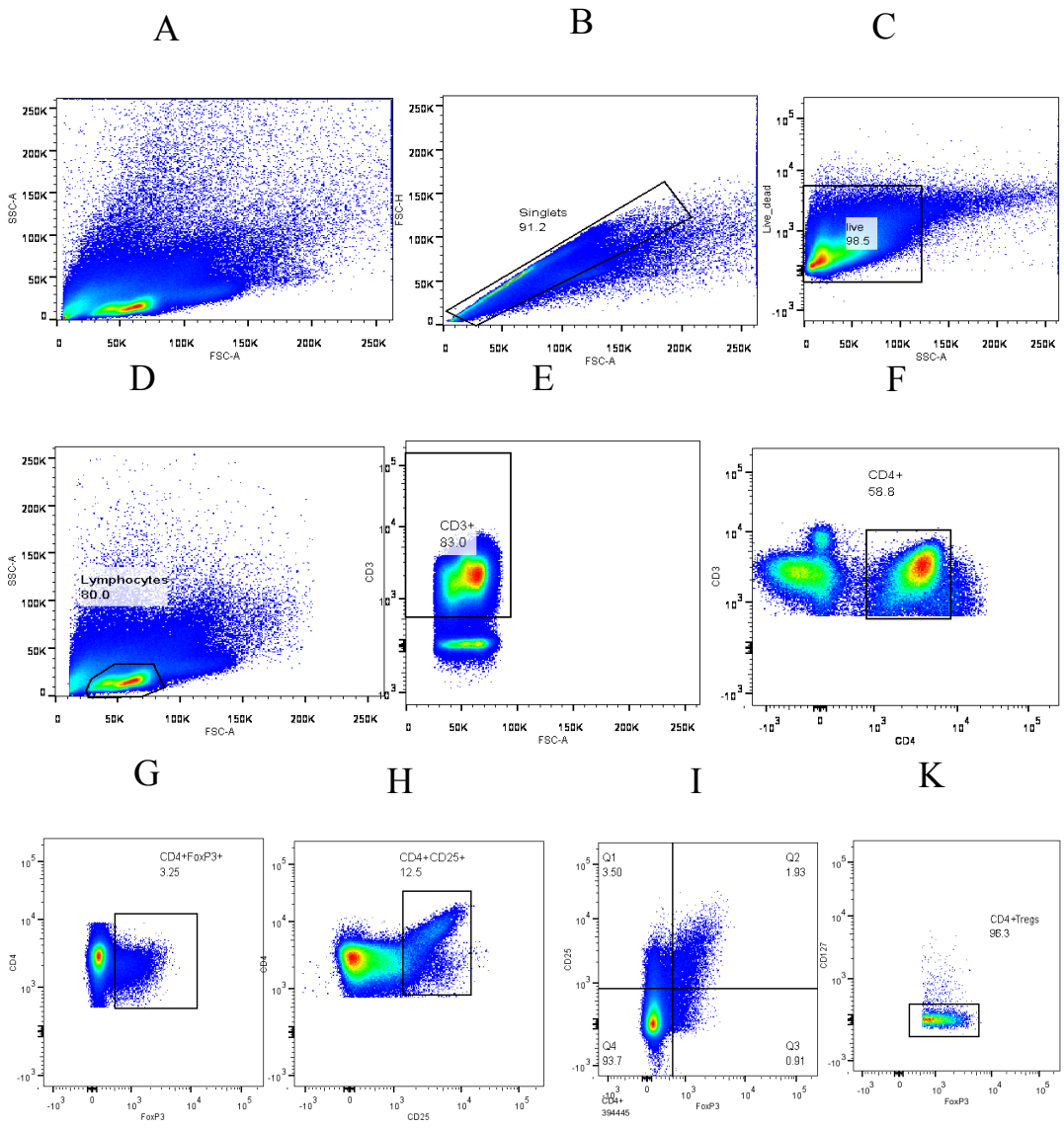


Figure 5.9. Gating for Tregs. A): SSCA versus FSCA of acquired cells on flow cytometry global worksheet, B) Siglets: obtained by gating FSCH versus FSCA. Cells with high FSCA are doublets or granulocytes, C): Live cells obtained by gating Live/dead aqua V500 vs SSCA. Live cells have low SSCA, D): lymphocytes obtained by gating SSCA versus FSCA. E): CD3 positive cells gated against FSCA, F): $CD4^+$ cells gated on $CD3^+$ T-cells, G): $FoxP3^+$ cells gated on $CD4^+$ T-Cells, H): $CD25^+$ cells gated on $CD4^+$T-cells, I): CD25 and FoxP3 gated on $CD4^+$ T-cells, K): CD127 gated with $CD25^+$ $FoxP3^+$ cells (Quadrant II of I). Then the $CD25^+FoxP3^+$ $CD127^{lo/-}$ cells were taken as $CD4^+$ Tregs ($CD4^+CD25^+FoxP3^+CD127^{lo/-}$).

5.3.9.2. Gating Memory T cells

Viable cells and lymphocytes were obtained as describe above under gating Tregs. The $CD3^+$ population were gated for $CD4^+$ and $CD8^+$ T-cells. On each population ($CD3^+$, $CD4^+$ and $CD8^+$ cells) CD45RO and CD62L were gated to classify into subpopulations of memory T cells. CD45RO is used to delineate between naïve and memory T-cells while CD62L is used to obtain activated T-cells. The combination of CD45RO and CD62L categorizes the population into naïve, central memory, activated memory and effector T- cells (Lugli et al., 2013) (Figure 5.10).

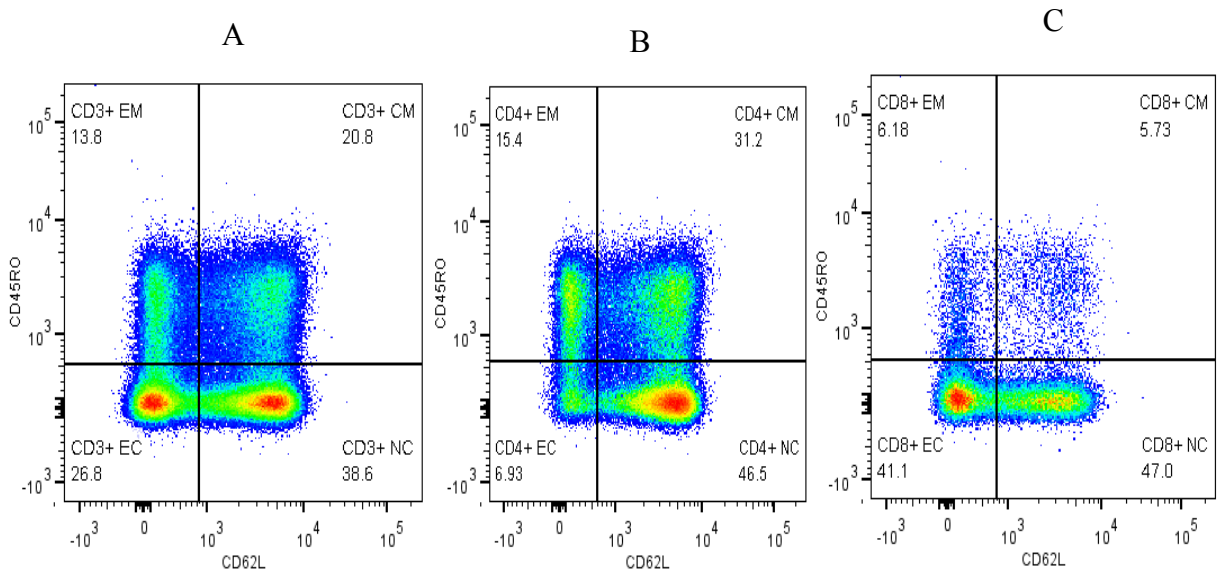


Figure 5.10. Gating memory T-cells on different cell types. A) gated on $CD3^+$ T-cells, B) gated on $CD4^+$ T-cells, C) gated on $CD8^+$ T-cells

5.3.9.3. Gating B cells

Singlets, viable cells and lymphocytes were obtained in the same way as described in the above sections. B-cells were separated from other lymphocytes by gating the lymphocytes with FSCA versus CD19. Then $CD19^+$ B-cells were gated with CD10 and $CD10^+$ B-cells were excluded from further gates since CD10 is expressed on immature B-cells. $CD19^+CD10^-$ B-cells were gated for CD27 versus CD21 to classify them into naïve cells, resting memory, activated memory and tissue-like memory B-cells (Table 5.2). In each memory B-cells, the degree of cell death (apoptosis) and B

cells activation were measured using PD1 (CD279) and CD95 respectively (Demberg et al., 2012).

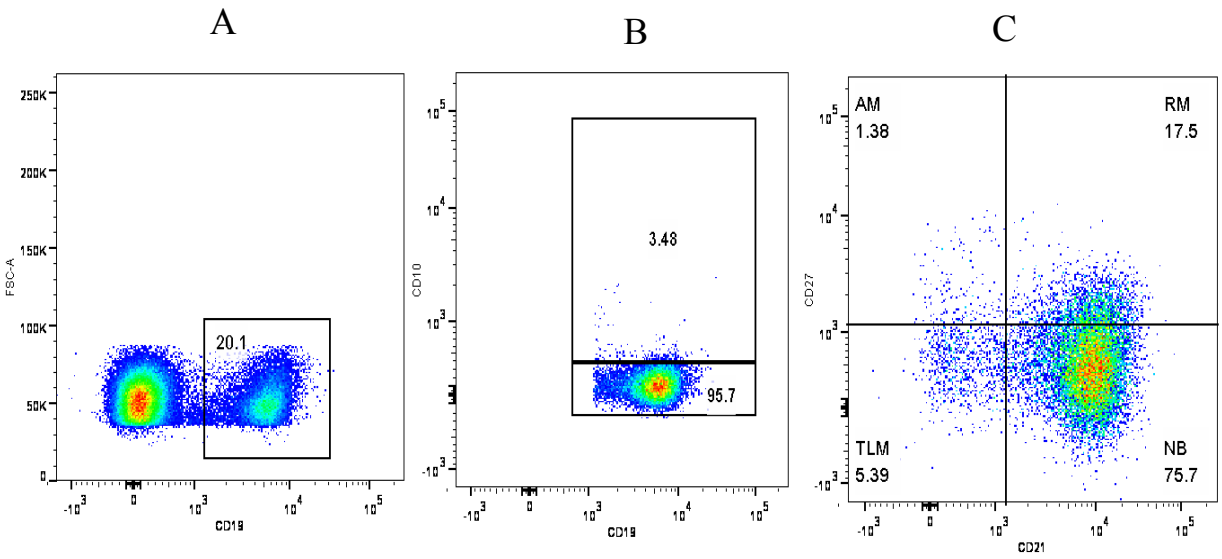


Figure 5.11. Gating strategy for B-cell sub populations; A) Gating B-lymphocytes by FSCA versus CD19, B) gating matured B-cells ($CD19^{+}CD10^{-}$), C) memory B-cells (obtained by gating matured B-cells for CD27 versus CD21).

Table 5.2. Memory T cells and their markers

Mature B cells (CD19+CD10-)	**Marker**	
	CD27	CD21
Naïve B cells (NV)	-	+
Resting Memory B cells (RM)	+	+
Activated Memory B cells (AM)	+	-
Tissue like memory B-cells (TLM)	-	-

SECTION 4: ENZYME- LINKED IMMUNOSORBENT ASSAY

5.4.1. Sample preparation

An enzyme-linked immunosorbent assay (ELISA) is a biochemical technique which is typically performed to detect the presence and /or amount of a target protein of interest within an experimental sample. A sandwich ELISA was used to measure the *in vitro* production of the cytokines (TNF-α, IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-17A) in culture supernatants of PBMCs from patients with ENL reactions and LL controls before and after treatment.

Sandwich ELISA is a special type of ELISA which measures antigens between two layers of antibodies (i.e. capture and detection antibody). The advantage of Sandwich ELISA is that the sample does not have to be purified before analysis, and the assay can be 2 to 5 times more sensitive than conventional ELISA (Gan and Patel, 2013). An ELISA well plate is coated with a known quantity of bound antibody to capture the desired antigen. After nonspecific binding sites are blocked using bovine serum albumin, the antigen-containing sample is applied to the plate. A specific primary antibody is then added that "sandwiches" the antigen. Enzyme-linked secondary antibodies are applied that bind to the primary antibody. Unbound antibody-enzyme conjugates are washed off. The substrate is added and is enzymatically converted to a colour that can be quantified using ELISA reader.

PBMCs thawing: During thawing, cells were transported in liquid nitrogen to a pre-adjusted water bath at 37ºC for 30 to 40 seconds until thawed half way and resuspended in 10% FCS in AIM-V at 37ºC containing 1/10,000 benzonase until completely thawed, washed 2 times (7 minutes each) and counted as described above.

Lymphocyte stimulation tests (LST): Total PBMCs (200,000 cells/well), were added in triplicate into 96 well U-bottom tissue culture plates and cultured with *M. leprae* whole cell sonicate (WCS; 10 mg/ml), phytohaemagglutinin (PHA; 1 mg/ml) or AIM-V medium at 37ºC with 5% CO_2 and 70% humidity. After 6 days, supernatants were collected and kept frozen until used in ELISA.

***M. leprae* whole cell sonicates (WCS):** Irradiated armadillo-derived *M. leprae* whole cells were probe sonicated with a Sanyo sonicator to 95% breakage. This material was kindly provided by Dr. J.S. Spencer through the NIH/NIAID ''Leprosy Research

Support'' Contract N01 AI- 25469 from Colorado State University (now available through the Biodefense and Emerging Infections Research Resources Repository listed at (http://www.beiresources.org/TBVTRMResearch materials/tabid/1431/Default.aspx).

5.4.2. The Cytokine ELISA protocol

We used a Ready-Set-Go! ® Sandwich ELISA for detection and quantification of the cytokines mentioned above in the supernatants obtained by stimulating the PBMCs from patients with ENL and LL controls with *M. leprae* WCS. A 96-well flat-bottom Nunc MaxiSorp® ELISA plates (Affymetrix, eBioscience, UK) were coated with 100µl/well of capture antibody for each cytokine in 1x coating buffer. The plates were sealed and incubated overnight at 4^{o}C. After aspiration, the plates were washed with 350µl/well-washing buffer three times with 1-minute soaking interval using microplate washer (Bio-Tek). Wells were blocked with 200µl/well of 1x ELISA diluents (Affymetrix, eBioscience, UK) and incubated for one hour at room temperature and followed by washing the plates once. A twofold serial dilution of a standard was prepared for each cytokine and 100µl/well of the serially diluted standard was pipetted to the first two columns of the plate in duplicates. To the remaining wells test samples (100µl/well) were added. A positive and negative control was included to each plate and then the plates were sealed and incubated for 2 hours at room temperature. After 2 hours incubation, the plates were aspirated and washed 3 times with 1-minute soaking interval as described above. After blotting the plates, a detection antibody (100µl/well) was added, sealed and incubated for 1 hour at room temperature. After one-hour incubation, the plates were aspirated and washed as described. A 100µl/well of avidin-horseradish peroxidase (Avidin-HRP) was added to each well and followed by incubation for 30 minutes at room temperature. Then the plates were aspirated, washed as described. A chromogenic substrate, Tetramethylbenzidine (TMB, 100µl /well) was added and incubated for 15 minutes in the dark. After 15 minutes incubation, 50µl/well stop solution (1 normal phosphoric acid, 1N H_3PO_4) was added to each well and the optical density (OD) at 450 nm was measured using an ELISA plate reader (Microplate reader; Bio-Rad, Richmond, CA). A curve fit was applied to each standard curve according to the manufacturer's manual. Sample concentrations were interpolated from these standard curves. Analyte concentrations outside the upper or lower limits of quantification were assigned the values of the

limits of quantification of the cytokine or chemokine. The standard curve plots are given in the Appendix 8.

Table 5.3. Antibodies used for ELISA

Cytokine	Assay Sensitivity	Standard curve range	Sample dilution	ELISA kit source	Cat no.
IL-6	2pg/mL	2-200pg/mL	1:10	ebioscience	88-7066
IL-8	2pg/mL	2-250pg/mL	1:10	ebioscience	88-7086
IL-10	2pg/mL	2-300pg/mL	1:1	ebioscience	88-7106
IL-17A	2pg/mL	2-200pg/mL	1:1	ebioscience	88-7170
TNF-α	4pg/mL	4-500pg/mL	1:2	ebioscience	88-7346
IFN-γ	4pg/mL	4-500pg/mL	1:2	ebioscience	88-7316
IL-1β	4pg/mL	4-500pg/mL	1:2	ebioscience	88-7010

5.4.3. Circulating Immune complex determination using C1-q ELISA

The human C1q ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human C1q. Circulating C1q forms together with the proenzymes C1r and C1s, the C1 macromolecule, the first component of the classical complement pathway. The formation of an antibody-antigen complex (immune-complex) is the principal way of activating the classical pathway of the complement system. With the Fc regions of IgG and IgM antibodies present in immune-complexes, efficient activation of the classical pathway is initiated. In this way, C1q acts to bridge the innate and adaptive immune systems (Leffler et al., 2014).

In C1-q ELISA, an anti-human C1q coating antibody is adsorbed onto microwells and human C1q present in the sample binds to the antibodies adsorbed to the microwells. After incubation, the unbound biological components are removed during wash step and a biotin-conjugated anti-human C1q antibody is added that binds to human C1q captured by the first antibody. After incubation with streptavidin-HRP, a coloured product is formed in proportion to the amount of C1q present in the sample following the addition of substrate solution.

For quantitative detection of human C1q in the plasma samples of patients with ENL and LL controls, we used human C1q platinum ELISA (ready-to-use sandwich

ELISA) with a sensitivity of 0.08ng/mL. The procedure is briefly described as follow as: The microwell plate coated with monoclonal antibody to human C1q was aspirated and washed twice with wash buffer. Then 100µl of serially diluted C1q standard was pipetted to the first two columns of the microwell strips. To the remaining strips pre-diluted to 1:1000 plasma samples (100µl/well) were added and to the last two wells a blank (assay buffer) was added as a negative control. The plate was sealed and incubated at 22ºC for 2hrs on a microplate shaker set at 400 revolutions per minute (rpm). After 2 hours incubation, the plate was washed 6 times with wash buffer and tapped on the absorbent pad. To each well, 100µl of biotin-conjugated anti-human C1q antibody was added and incubated at 22ºC for 1hr on a microplate shaker set at 400rpm. Then the microplate was washed as described above and followed by the addition of 100µl Streptavidin-HRP to all wells. The plate was sealed and incubated at 22ºC for 1hr on a microplate shaker as described. After six washes 100µl of TMB substrate solution was added to all wells and incubated at 22ºC for 30 minutes in the dark. The colour development on the plate was monitored and the substrate reaction was stopped by pipetting 100µl of stop solution (1N H_3PO_4). The optical density (OD) at 450nm was measured using an ELISA plate reader (Microplate reader; Bio-Rad, Richmond, CA). A curve fit was applied to the standard curve according to the manufacturer's manual using Microplate Manager® 6 Software (Bio-Rad, Richmond, CA) and the unknown concentration of C1q in each sample was extrapolated from these standard curves (Appendix 9).

SECTION 5: REAL -TIME PCR ASSAYS

In the real-time polymerase chain reaction (qPCR) assay, the mRNA gene expression levels for pro-inflammatory cytokines/chemokines listed in the previous section have been determined in blood and biopsy samples obtained from patients with ENL and LL controls before and after treatment. The real-time PCR assay included mRNA isolation and quantification, cDNA synthesis and real-time quantitative PCR.

5.5.1. RNA isolation from PAXgen Blood RNA Tubes

PAXgene Blood RNA Kit was used to isolate RNA from blood stored in PAXgene Blood RNA Tubes. PAXgene Blood RNA tubes were brought to safety cabinet and allowed to reach room temperature (18-22^{o}C) for approximately two hours or overnight. After thawing, the tubes were carefully inverted 10 times. Then the PAXgene Blood RNA Tubes were centrifuged for 10 minutes at 5000g using a swing-out rotor. The supernatant was removed by decanting and 4ml RNase-free water (Qiagen) was added to the pellet and the tube was closed using a fresh secondary Hemogard™ (Qiagen) closure followed by vortex until the pellet completely dissolved and centrifuged for 10 minutes at 5000g. The supernatant was discarded and 350µl Buffer BR1 (Qiagen) was added. Then the sample was pipetted into a 1.5 ml RNase-free microcentrifuge tube (Star lab, Ltd., UK) followed by addition of 300µl Buffer BR2 and 40µl proteinase K (Qiagen) and mixed by vortex for 5 seconds and then incubated at 55^{o}C for 10 minutes on a shaker set at 400rpm. The lysate was directly pipetted into a PAXgene Shredder spin column (Qiagen) which was placed in a 2ml processing tube. The lysate then centrifuged for 3 minutes at maximum speed (20,000g). The entire supernatant of the flow-through was carefully transferred to a fresh 1.5ml microcentrifuge tube without disturbing the pellet in the processing tube. Then 350µl ethanol (100%, 200 proof, molecular grade, Sigma-Aldrich, UK) was added and centrifuged briefly (1-2 seconds at 1000g) to remove drops from the inside of the tube lid. Then, 700µl of the sample (resuspended in 350µl ethanol) was pipetted into the PAXgene RNA spin column (red) placed in a 2ml processing tube, and centrifuged for 1 minute at 20,000g. The old processing tube containing flow-through was discarded and the spin column was placed in a new 2mL processing tube. Three hundred and fifty microliters of Buffer BR3 (Qiagen) was pipetted into the PAXgene RNA spin column, centrifuged for 1 minute at 20,000g and the old processing tube

containing flow-through was discarded and the spin column was placed in a new 2mL processing tube. Then 10µl DNase I stock solution was added to 70µl Buffer RDD in a 1.5ml microcentrifuge tube and mixed gently by flicking the tube and centrifuged briefly to collect the residual liquid from the sides of the tube. DNase I incubation mix (80µl) was pipetted directly onto the PAXgene RNA spin column membrane (containing the sample) and placed on the bench-top at 20-30^{0}C for 15 minutes. Then 350µl Buffer BR3 was pipetted into the PAXgene RNA spin column and centrifuged for 1 minute at 20,000g. The old processing tube containing flow-through was discarded and the spin column was placed in a new 2mL processing tube. Five hundred microliter Buffer BR4 was added to the PAXgene RNA spin column and centrifuged for 1 minute at 20,000g followed by replacing the old processing tube as described and the procedure was repeated once but this time for 3 minutes. After replacing the old processing tube, without adding any buffer, the spin column was centrifuged for 1 minute at 20,000g. The tube containing the flow-through was discarded and followed by addition of 40µl Buffer BR5 directly onto the PAXgene RNA spin column membrane, centrifuged for 1 minute at 20,000g to elute the RNA and this step was repeated once. Then, the elute RNA was incubated for 5 minutes at 65^{0}C and followed by immediate chilling. Finally, the RNA yield was determined using a NanoDrop 2000, spectrophotometer (Thermo Scientific, Epsom, UK). For all samples cDNA was synthesized on the same day to avoid the risk of RNA degrades during storage.

5.5.2. RNA isolation from RNAlater skin biopsy samples

RNA was isolated from skin biopsy samples stored in RNAlater™ (Ambion, Austin, Texas) using the RNeasy Fibrous Tissue Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. Four-millimetre punch skin biopsy (approximately 15mg) was disrupted in 300µl buffer RLT (Qiagen, Crawley, UK) using a disposable pellet and pestle (Anachem, Luton, UK) and homogenised with a Qiashredder (Qiagen, Crawley, UK). DNA was digested with DNase I (Qiagen, Crawley, UK). After homogenization, 590µl RNase-free water and 10µl proteinase K was added to the homogenised tissue followed by incubation in a water bath at 55^{o}C for 10 minutes. The remaining procedures were similar with the procedures described under RNA extraction from PAXgene Blood RNA Tubes. After determining the yield and quality of RNA, cDNA was synthesised on the same day and stored at-20^{o}C.

5.5.3. cDNA synthesis

Complementary DNA (cDNA) was synthesised from RNA using High Capacity cDNA Reverse Transcriptase Kit (AB Applied Biosystems, UK). Reactions consisted of 1x RT buffer, 0.5 mM dNTP, 1µM Oligo-dT primer, 0.5 units/µl RNase inhibitor (all from AB Applied Biosystems, UK), 2.5units/µl reverse transcriptase, 80ng (1µl) template RNA and nuclease- free water to a total volume of 20µl. Reactions were incubated in an ABI9700 Programmable Thermal Cycler (Applied Biosystems, Foster City, California) for 10 minutes at 25ºC followed by 120 minutes at 37ºC and 5 minutes 85ºC then and cooling to 4 ºC.

5.5.4. Primers

Genomic DNA and mRNA sequences were obtained from the National Centre for the Biotechnology Information (NCBI) data bank. Primers between 20-24 nucleotides in length were designed across intron/exon boundaries on mRNA sequence to give a product of 100-500bp (Table 6.4). These primers could not amplify genomic DNA targets and functioned with cDNA templates only as they spanned exon boundaries.

All primer sequences were blasted on the NCBI data bank to confirm their specificity. Custom synthesis of oligonucleotide primers was performed by Sigma-life science and provided in desalted form. Primer stock solution was prepared according to the manufacturer's recommendation. A working stocks of 10.0µM were prepared and kept at -20 °C until used (Table 5.4).

Table 5.4. Primer sequences, length, melting temperature, amplicon size, %GC ratio and NCBI accession number.

Primer (F, forward; R, reverse)	Sequence (5' to 3')	Length	T_m (ºC)	% GC ratio	Amplicon size (bp)	Accession number
IL-10 FW	TGAGAACCAAGACCCAGACA	20	63.4	50	182	
IL-10 RV	TCATGGCTTTGTAGATGCCT	20	62.2	45		NM_000594
TNF-a FW	AGCCCATGTTGTAGCAAACC	20	58.74	50.00	104	
TNF-a RV	GCTGGTTATCTCTCAGCTCCA	21	59.24	52.38		NM_172369
IL-17A FW	AGACCTCATTGGTGTCACTGC	21	64.2	52.3	238	
IL-17A RV	CTCTCAGGGTCCTCATTGCG	20	67.2	60		NM_002190
IL-6 FW	TTCGGTCCAGTTGCCTTCTC	20	66.5	55	193	
IL-6 RV	TACATGTCTCCTTTCTCAGGGC	22	64.3	50		NM_000600
IL-1B FW	AGCCCCAGCCAACTCAATTC	20	67.4	55	380	XM_006712496
IL-1B RV	CATGGAGAACACCACTTGTTGC	22	66.6	50		
Fox P3 FW	TGGAGAAGGAGAAGCTGAGTGC	22	61.99	54.55	73	XM_011543919
FoxP3 RV	ACAGATGAAGCCTTGGTCAGTGC	23	63.24	52.17		
IFN-g FW	GGCTTTTCAGCTCTGCATCG	20	59.90	55.0	172	
INF-g RV	TCTGTCACTCTCCTCTTTCCA	21	57.76	47.62		NM_000619
IL-8 FW	ACCGGAAGGAACCATCTCAC	20	59.39	55.0	104	
IL-8 RV	AAACTGCACCTTCACACAGAG	21	58.71	47.62		NM_000584
HuPO FW	GCTTCCTGGAGGGTGTCC	18	59.33	66.67	105	
HuPO RV	GGACTCGTTTGTACCCGTTG	20	58.86	55.00		NM_001002
TGF-β FW	ACATCAACGCAGGGTTCACT	20	59.89	50	264	XM_011527242
TGF-β RV	GAAGTTGGCATGGTAGCCC	20	60.03	55		
C1-qA FW	ATGGTGACCGAGGACTTGTG	20	59.68	55	276	NM_015991
C1-qA RV	GTCCTTGATGTTTCCTGGGC	20	58.82	55		
C1-qB FW	CAGGTTGAAATCAGCATTGCC	21	58.39	47.62	163	XM_011542059
C1-qB RV	CTGTGTCAGACGCCTCCTTTC	21	60.94	57.14		
C1-qC FW	AAGGATGGGTACGACGGACTG	21	61.56	57.14	213	NM_172369
C1-qC RV	TTTCTGCTTGTATCTGCCCTC	21	58.01	47.62		

5.5.5. Choice of the control gene (HuPO)

Human Acidic Ribosomal Protein (HuPO) has been previously described in PCR experiments examining the housekeeping gene expression in whole blood and PBMCs samples with minimum variability compared to other housekeeping genes such as glaceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and hypoxanthine phosphoribosyltransferase (HPRT) (Dheda et al.,2004). Hence, Human Acidic Ribosomal Protein (HuPO) has been chosen as a house-keeping gene in our quantitative PCR experiment.

5.5.6. Real-time quantitative PCR

Real-time quantitative PCR for all genes was performed on the Rotor-Gene™ 3000 programmable thermal cycler (Corbett Life Science (Qiagen), Crawley, UK) using Roter-gene® SYBR® Green PCR Kit (Qiagen, Crawley, UK). The Rotor-Gene SYBR Green PCR Kit enables rapid and reliable real-time PCR quantification on the Rotor-Gene Q without the need for optimization of reaction and cycling conditions. In our experiment, we optimized the T_m values for each primer pair.

PCR reactions consisted of 1x Rotor-gene SYBR Green PCR Master Mix, 1µM forward primer, 1µM reverse primer, 1µl cDNA (approximately 100ng) and nuclease-free water to a total volume of 12.5 µl. The master mix contained HotStarTaq Plus DNA polymerase, Rotor-gene SYBR Green PCR buffer, dNTP mix and SYBR Green I fluorescent dye.

The Rotor-Gene conditions were set as follows: Initial activation step (polymerase activation) was achieved by incubating at 95°C for 15 minutes, 40 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 10 seconds, extension at 72°C for 20 seconds and fluorescence acquisition for 5 seconds at 72°C.

The primer-dimer formation was checked by melting curve analysis. Melting point data were obtained by increasing the temperature from 50°C to 99°C by 1°C on each step. The interval between increases in temperature was 30 seconds for the first step and then 5 seconds for subsequent steps.

An assay control was included from mRNA extraction to the amplification steps. For mRNA extraction, one assay control per batch was used. The assay control included all buffers except the sample and was processed under identical conditions with the samples. The same assay control was used during cDNA synthesis and real-time quantitative PCR.

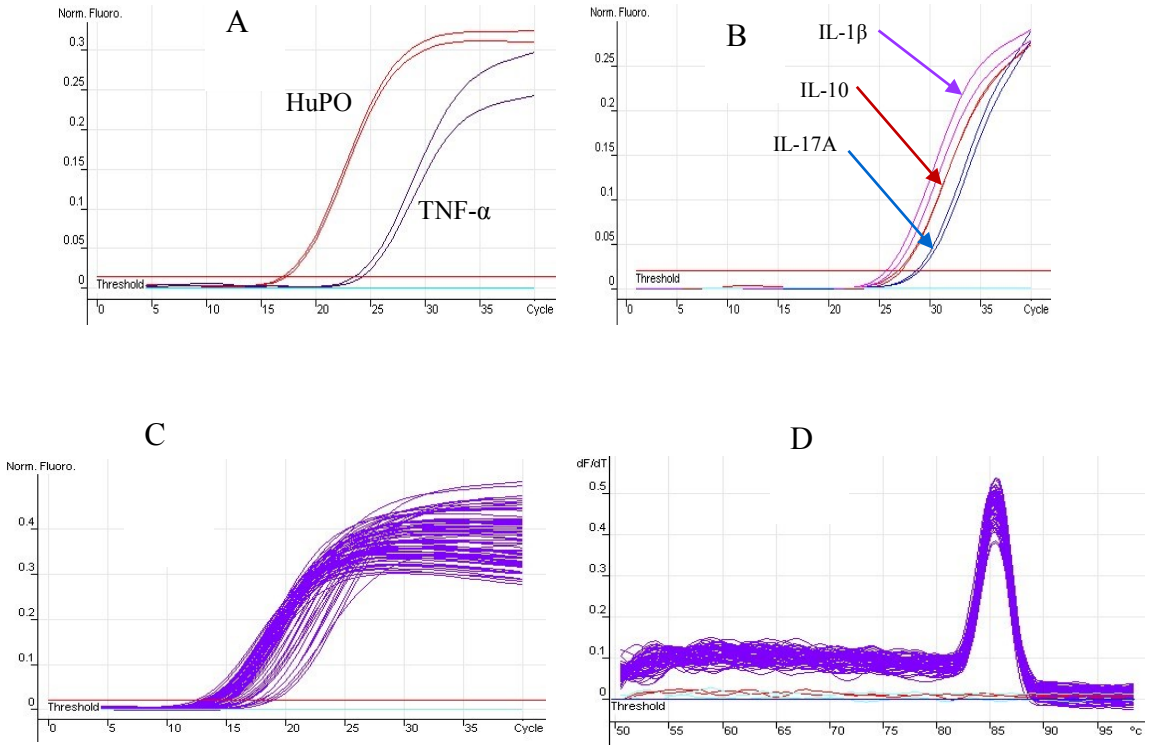


Figure 5.12. PCR optimization: A) shows the optimizationle threshold or house keeping gene (HuPO) and TNF-α, B) shows the optimization cycle threshold for IL-1β, IL-10 and IL-17A, C) shows the cycle threshold of house keeping gene for multiple samples, D) showe the melt cuve analysis. The peak curve indicates the primer amplified the same region of all samples and primer dimer is not detected as there is only one such peak.

5.5.7. Calculating the relative gene expression

The relative gene expression was analyzed by using the $2^{-\Delta\Delta CT}$ method (Cikos et al., 2007). The C_T value is the threshold number for the amplification of the target gene. Cikos et al. compared the six different methods currently used for real-time PCR data analysis and has shown that the best results were obtained with the relative standard curve (ΔC_T) method and the least coefficient of variation (ΔCV).

The C_T values were abtained for the target gene and control gene (HuPO) for each patient sample at each time point. Then, the difference in C_T value was obtained by subtracting the C_T of the target gene from the C_T of the control gene and designated as ΔC_T. To compare the target gene expression in patients with ENL and LL controls, $\Delta\Delta C_T$ was obtained by subtracting the ΔC_T of LL patient control from the ΔC_T of the patient with ENL. Then, the fold change was obatined by using the formula $2^{-\Delta\Delta CT}$. similarly, for the comparison of the relative target gene expression in patients with ENL before and after treatment, $\Delta\Delta CT$ was obtained by ΔC_T (after) minus ΔC_T (before). Then the fold change for target gene expression from the baseline (before treatment) was given by $2^{-\Delta\Delta CT}$.

SECTION 6: STATISTICAL METHODS

5.6.1. Data recording and management

All clinical data were recorded on standardized patient record specifically designed by the ENL International STudy Group (ENLIST). The forms were kept in a separate set of case notes from the usual clinical records. All study records were kept in a locked box accessed only by the principal investigator and the study coordinator. For follow-up studies, a special ID card was prepared for each patient with a specific code. Patients have been advised to bring these identification cards when coming to the hospital for any health complaints and notified to show to the study coordinator. An attendance log book was prepared to document any patient complaints. The information was reviewed, compiled and used during the write-up of the thesis.

With regard to the laboratory-based data, all data were recorded on laboratory notebook. Information which included the amount of blood obtained from each patient per visit, number of PBMCs isolated, percentage of cell viability, number of frozen PBMCs/vial, the number of vials containing frozen PBMCs, the number of cells recovered during thawing, samples used for staining, and others. A separate laboratory notebook were used for flow cytometry, ELISA and qPCR. In addition, all information was also recorded on Excel spreadsheet as an electronic data in two copies. One copy was used at the study site and the other copy stored on LSHTM data server through remote desktop from the field site. Updating data copy has been done every 48 hours.

5.6.2. Strategy for data analysis

Clinical Data: An excel spreadsheet double data entry was used for all data. Data cleaning was performed prior to analysis. Clinical data were analysed by SPSS version 20. Depending on the nature of the variable and the normality of the data, either parametric or non-parametric analysis was used. Categorical variables were analysed by non-parametric methods and normally distributed numerical variables with parametric methods. Whenever mean is used for comparison, data presentation has followed the form of mean ± standard error of the mean (SE).

Flow cytometry data: Flow cytometry data analysis involves several steps. During sample acquisition on BD FACSCanto II, preliminary statistical data are displayed by the BD FACSDiva™ Software that it is used for monitoring the overall performance

of the cytometer during data acquisition. In flow cytometry, the intensity of a distribution of the dots can be represented by the position of the "centre" of the distribution (usually median) and the spread of the distribution by the coefficient of variation (CV). These dots are gated into sub-populations based on their side scatter (SSC) and forward scatter (FSC) and the specific fluorochromes used by using Flowjo. For gating FACS data by flowjo version10, into subpopulations logicle (bi-exponential) method was selected as recommended by Herzenberg et al. (2006). Then the relative percentage of each subpopulation was copied to Excel for each sample and finally an excel spreadsheet electronic data was generated and used for subsequent statistical analysis. STATA 14 version 2 software was used for data analysis and Prism 6 version 4 software to produce graphs. Mann-Whitney and Kruskal-Wallis tests were performed depending on the number of variables needed for comparison. Median and Hodges–Lehmann estimator were used for result presentation. Hodges–Lehmann is used to measure the effect size for non-parametric data (Gail, 2002).

ELISA Data: The optical density of each sample for each cytokine was obtained by the ELISA reader. The OD was converted to concentration (pg/mL) by microplate manager 6. Unpaired t-test was used to compare the relative concentration of the cytokines production in patients with ENL and LL controls. For comparing the cytokine concentration in patients with ENL before and after treatment, paired t-test was used. Results are presented as mean ± standard error of the mean with P-values.

Real-time quantitative PCR: for the mRNA gene expression of target genes, the relative threshold cycle value (C_T) comparison method was used as previously described in section 5. The fold change of each target gene was used for statistical analysis. Unpaired t-test was used to compare the fold change of each target gene for patients with ENL compared to LL patient controls. To compare the expression level of the desired gene in patients with ENL before and after treatment, paired t-test was used.

5.6.3. Data presentation

Clinical data: The results of clinical data were presented in the form of figures and tables depending on the nature of the data.

Flow cytometry data: considering the complexity of flow cytometry data, graphical methods of data presentation was preferred to tabular methods. Tables and other flow cytometry representative results are annexed.

Real-time quantitative PCR: The results of real-time quantitative PCR are presented in tables. Tables are preferred to figures since fold changes and $\Delta\Delta C_T$ are best presented and easily understandable by tables. If the value $\Delta\Delta C_T$ is negative, it indicates upregulation of the gene expression whereas positive $\Delta\Delta C_T$ value means the downregulation of the gene expression for that particular target gene.

CHAPTER 6: RESULTS

An Overview

The results of this study are presented in chapter 6 and it is divided into nine sections. Section one deals with socio-demographic and clinical backgrounds of study subjects. Clinical pictures and histopathological features of patients with ENL and LL controls are presented in section one. Section two deals with the results for T-cell regulation in patients with ENL reaction and LL controls before, during and after treatment as well as within ENL cases before and after treatment. These results included in this section are: the median percentages of $CD4^+$ and $CD8^+$ T-cells, $CD4^+/CD8^+$ T-cells ratio, $CD4^+FoxP3^+$, $CD8^+FoxP3^+$, $CD4^+CD25^+$, $CD8^+CD25^+$, $CD4^+CD25^+FoxP3^+$, $CD8^+CD25^+FoxP3$ T-cells, $CD4^+$ and $CD8^+$ Tregs. In section three the results for memory T- cells and T-cell activation in the PBMCs from patients with ENL and LL controls before, during and after treatment are described. In section four the results of regulatory and memory T-cells in acute and chronic ENL are presented.In section five, results obtained from B-cell phenotyping are presented. The proportion of matured B-cells, naive B-cells, resting memory B-cells, activated memory B-cells and tissue-like memory B-cells in the PBMCs from patients with ENL and LL controls are presented in this section.

The cytokine results are presented in section six. The *in vitro* cytokine production in the PBMCs from patients with ENL and LL controls in response to *M.leprae* whole-cell sonicates before and after treatment are presented in this section. In section seven, the mRNA gene expression levels of selected pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-6,IL-8,IL-17A) and regulatory cytokines (IL-10, TGF-β) as well as the transcription factor, FOXP3 in whole blood and skin biopsy samples from patients with ENL and LL controls before and after treatment are presented. The results of the C1q ELISA is presented in section eight. Results obtained for the gene expression levels of C1q are also presented in section eight. Section nine presents the levels of anti-PGL-1, LAM, and Ag85 antibodies in the plasma samples of patients with ENL and LL controls before and after treatment.

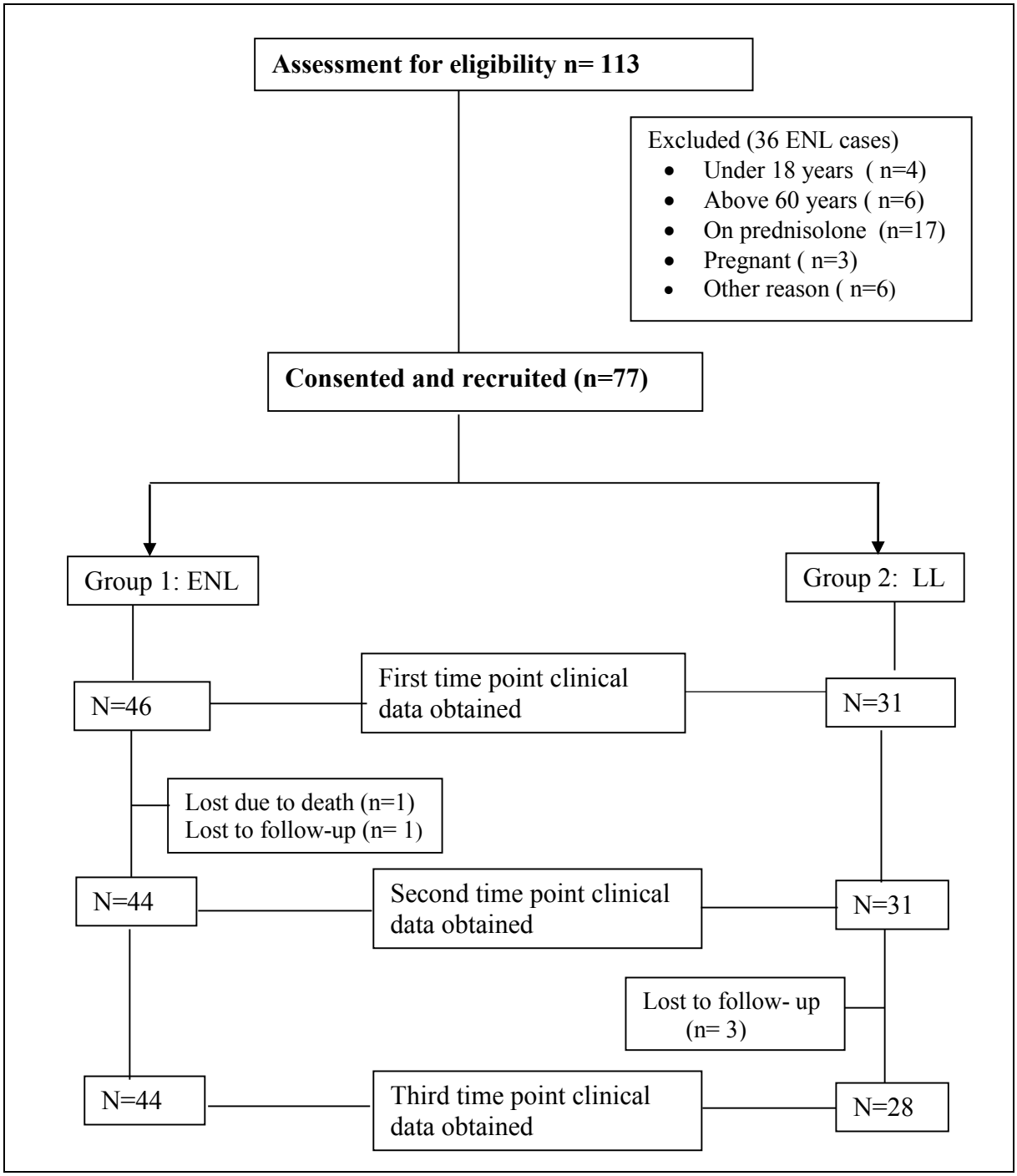


Figure 6.1.1. Flow diagram for study subjects recruitment and follow-up

SECTION 1: SOCIO-DEMOGRAPHIC AND CLINICAL CHARACTERISTICS

1.1. Socio-demographic and clinical backgrounds of study subjects

Clinical data were obtained on 77 patients (46 LL patients with ENL reactions and 31 non-reactional LL patients) at recruitment (Figure 6.1.1). The male to female ratio was 2:1 with a median age of 27.5 [range: 18-56] years in patients with ENL and nearly 3:1 with a median age of 25.0 [range: 18-60] years in patients with non-reactional LL controls. The median age was slightly higher in patients with ENL than in LL controls. Interestingly, the age range of females in both groups was relatively narrow (18-35 years) compared to males (18-60). More than half of the patients with ENL were previously treated with MDT. Half of the patients with ENL had acute ENL at the time of enrolment with mean BI 3.9 ±0.205 SE (standard error). Recurrent ENL cases had the highest mean BI (4.9 ±0.409 SE) at leprosy diagnosis whereas acute and chronic cases had comparable mean bacterial index (BI). All patients were negative in the HIV test before and after treatment (Table 6.1.1).

Pain was the most common symptom reported by patients with ENL. All patients with ENL reported that they had pain at the time of enrolment. About 80% of the patients with ENL had reported skin pain and more than 70% had nerve and joint pains during enrolment. Other pain sites reported include bone, digits, eyes, muscles, lymph nodes and testes (Figure 6.1.2).

Table 6.1.1. Sociodemographic and clinical characteristics of study subjects at enrolment

Variables		ENL (n=46) n (%)	LL (n=31) n (%)
Sex	Male	31 (67.4)	23 (74.2)
	Female	15 (32.6)	8 (25.8)
Median age in years (range) group		27.5 (18-56)	25.0 (18-60)
Median age in years (range) Male		28 (18-56)	26.0 (18-60)
Median age in years (range) Female		26.7 (18-35)	21.0 (18-30)
MDT status	No previous MDT	10 (21.7)	23 (74.2)
	Current	9 (19.6)	7 (22.6)
	Completed	27 (58.7)	1 (3.2)
HIV status	Positive	0 (0.0)	0 (0.0)
	Negative	46 (100.0)	31 (100.0)
Duration of current ENL symptom (Episode) Mean ± SE [days]		6.8 ±0.491 (range: 1-15)	-
Clinical status at recruitment			
ENL type	Acute	23 (50.0)	-
	Recurrent	5 (10.9)	-
	Chronic	18 (39.1)	-
LL type	New	-	23 (74.2)
	Relapse	-	5 (16.1)
	Defaulter	-	3 (9.7)
BI at diagnosis, Mean ± SE (range)			
ENL	Acute	3.9 ±0.205 (2-6)	-
	Recurrent	4.9 ±0.409 (4-6)	-
	Chronic	3.7 ±0.103(3-4)	-
LL	Untreated (new)		4.1 ±0.259 (2-6)
	Relapse		4.2 ±0.330 (4-5)
	Defaulter		4.9 ±0.150 (4-5)

Fever was reported in 31 (71.7%) patients with ENL while skin lesions were reported nearly in all of these patients (95.7%). Sixteen (34.8%) patients with ENL complained depression and 47.8% nasal stuffiness. Other reported symptoms included oedema, insomnia, anorexia, weight loss, joint swelling and malaise (Figure 6.1.3).

The morphology of cutaneous lesions showed that 95.7% individuals had nodular lesions, about two-third had subcutaneous nodules and a quarter of patients had scar due to ENL. While one-third of the patients had ulcerated lesions, only 4% had necrotic lesions. Eight patients (17.3%) had vesicles, bullae or pustular lesions (Figure 6.1.4).

In most patients with ENL (73.9%), the number of skin lesions recorded at the time of enrolment was 11-50. Few patients had five or less skin lesions. Almost all patients (97.8%) had skin lesions on the upper limbs. A considerable number of the patients also had skin lesions on the lower limbs (95.7%) and on the head and neck (63.0%). Half of the patients reported reduced nerve sensation. Paraesthesia and hyperaesthesia were reported by 13% and 23.9% patients respectively (Table 6.1.2).

More than half (52.2%) of patients with ENL had old nerve function impairment (NFI) while 13% had new NFI at the time of enrolment. Facial and limb oedema were the most reported organs involved in ENL reactions. Facial oedema was reported in 56.5% of the patients with ENL and nearly half (47.8%) of the patients had oedema on their lower limbs. Other organs involved in the patients with ENL were small joint arthritis (28.3%), large joint arthritis (15.2%), conjunctivitis (4.3%), lagophthalmos (2.2%), scleritis (8.7%), lymph node (15.2%) and dactylitis (2.2%) (Table 6.1.2).

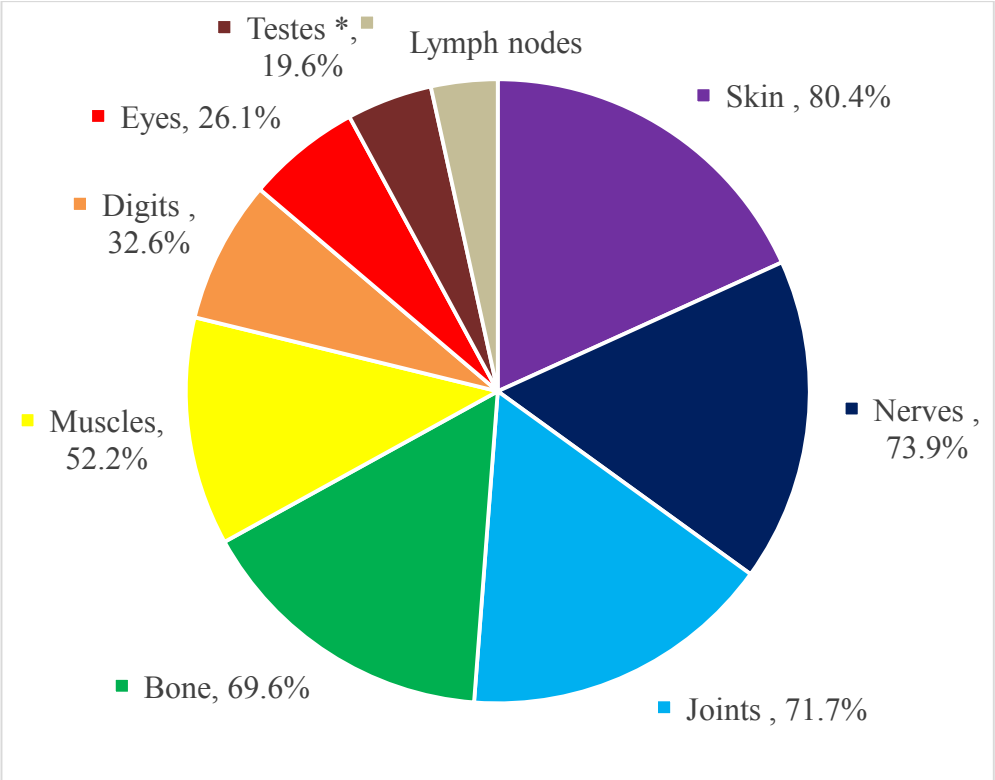


Figure 6.1.2. Location of pain in the patients with ENL. *value is among 31 males

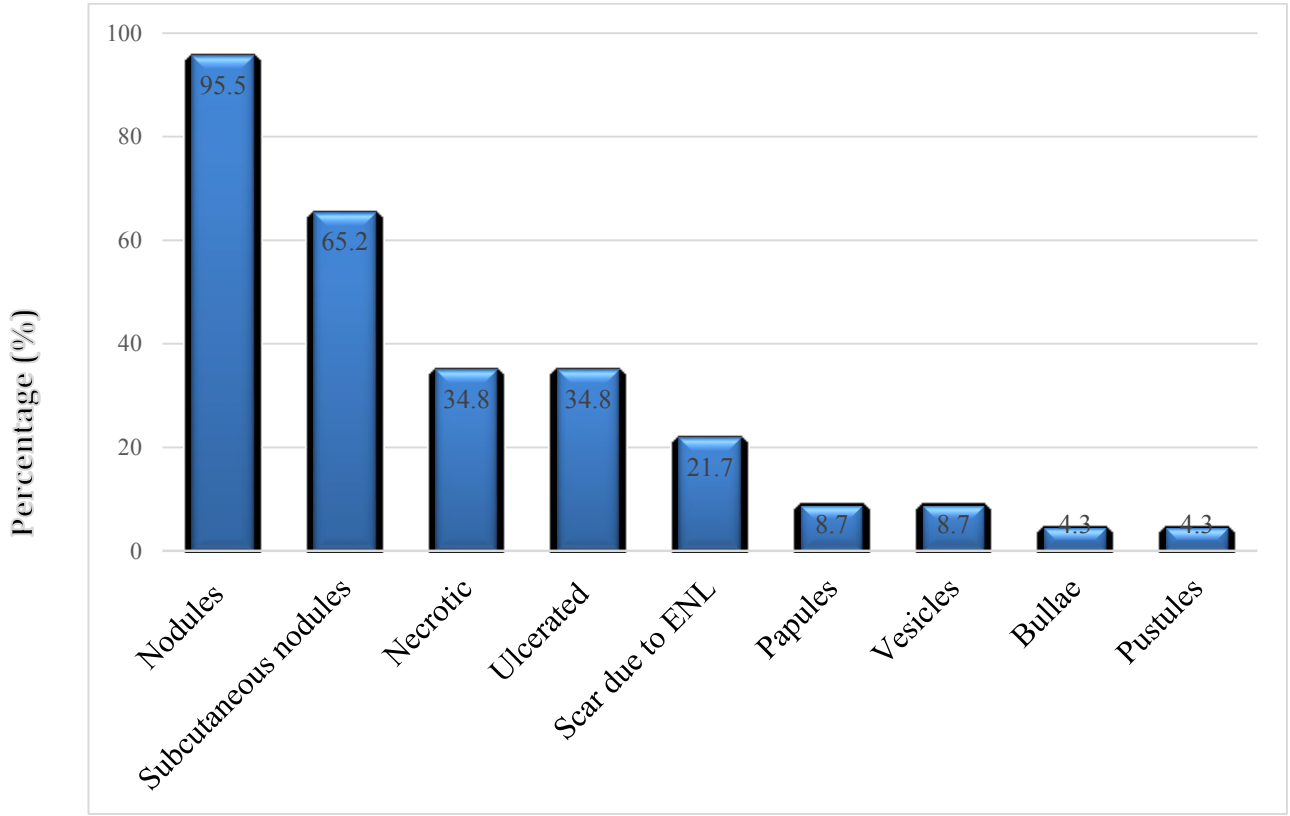


Figure 6.1.3. Frequency of the morphology of skin lesions in patients with ENL

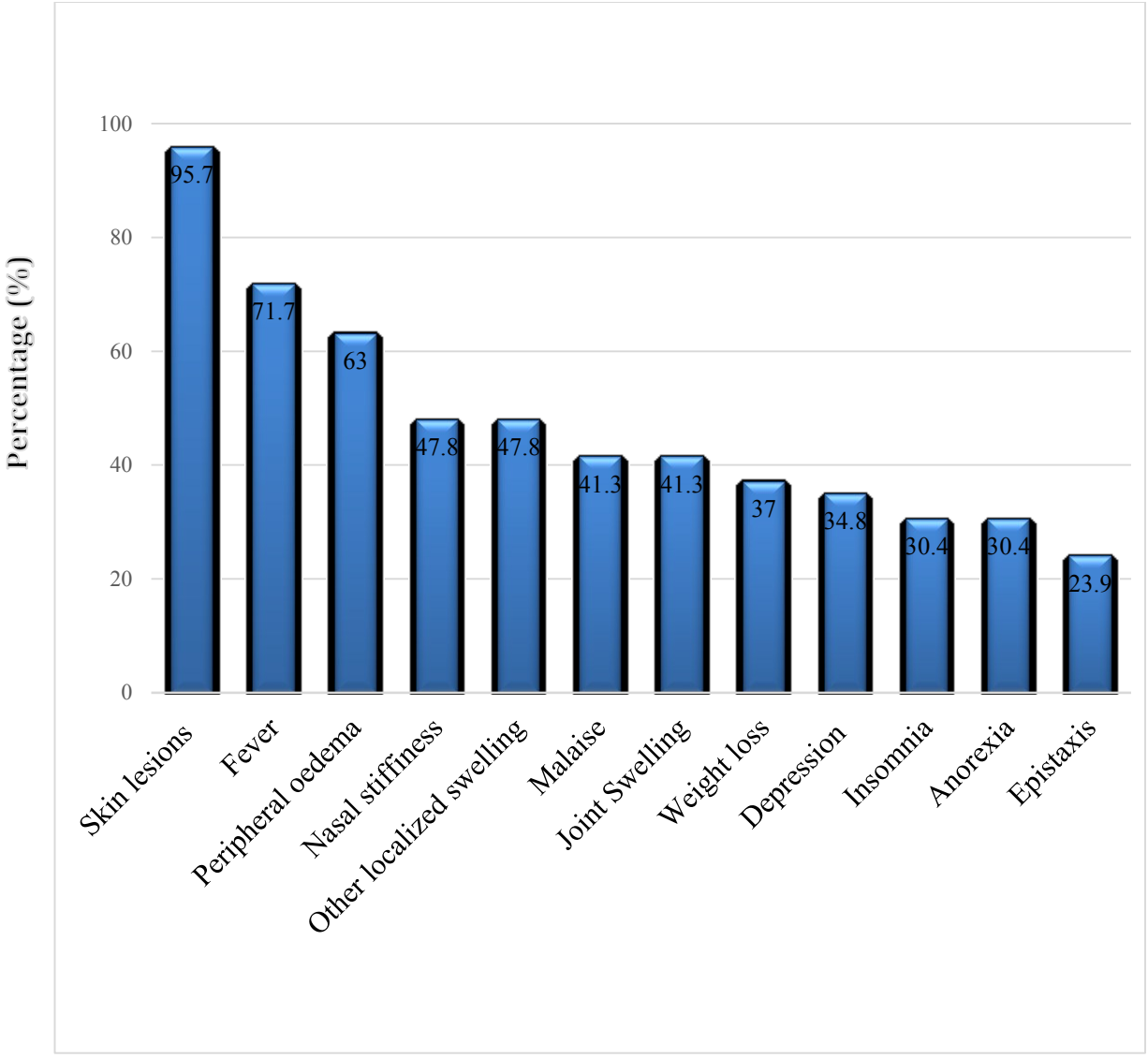


Figure 6.1.4. Symptoms other than pain in patients with ENL

Table 6.1.2. Other clinical pictures in patients with ENL at enrolment

		number	%
Number of skin lesions	<5	3	6.5
	6-10	4	8.7
	11-20	18	39.1
	21-50	16	34.8
	>50	5	10.9
Location of skin lesions	Head/neck	29	63.0
	Trunk	18	39.1
	Upper limbs	45	97.8
	Lower limbs	44	95.7
Nerve symptoms	Reduced Sensation	23	50.0
	Paraesthesia	6	13.0
	Hyperaesthesia	11	23.9
	Weakness	35	76.1
Nerve function impairment (NFI)	Old	24	52.2
	New	6	13.0
Organs involved in ENL			
Oedema	Hand	26	56.5
	Face	16	34.8
	Lower limbs	22	47.8
Dactylitis		1	2.2
Large joint Arthritis		7	15.2
Small joint arthritis		13	28.3
Conjunctivitis		2	4.3
Lagophthalmos		1	2.2
Scleritis		4	8.7
Lymph node		7	15.2

1. 2. Histopathological features of study subjects

Paraffin- embedded sections of skin biopsy samples from ENL and LL lesions were graded by a histopathologist. Before treatment dermal neutrophil infiltration was diagnosed in 58.8% of patients with ENL cases compared to 14.3% in LL patient controls and the difference was statistically significant (P=0.004). Lymphocyte infiltration was recorded in all ENL and LL lesions. Foamy histiocytes were more frequently seen in LL lesions (95.3%) than in ENL lesions but the difference was not statistically significant at recruitment. Panniculitis was diagnosed only in 62.5% of lesions from patients with ENL reactions. After treatment, while neutrophils were present in 5 biopsies from patients with ENL reactions, lymphocytes were seen in 20 skin biopsies of patients with ENL (Table 6.1.3). Flat granular PMN infiltration with perivascular lymphocyte infiltration and lobar panniculitis were diagnosed as defining features of ENL lesions compared to reticular dermal infiltration of lymphocytes and flat epidermis for LL lesions (Figure 6.1.5).

Table 6.1.3. Histopathological features of study subjects before and after treatment

Diagnosis	Before treatment n (%)			After treatment n (%)		
	ENL n= 34)	LL (n=21)	(P-value)	ENL (n=33)	LL (n=21)	P-value
Neutrophil infiltration	20 (58.8)	3 (14.3)	0.004*	5 (15.2)	7 (33.3)	0.058
Lymphocytes	34 (100.0)	21 (100.0)	0.984	20 (60.6)	21 (100.0)	0.006*
Foamy histiocytes	29 (85.3)	20 (95.3)	0.627	14 (42.2)	18 (85.7%)	0.001*
Eosinophils and mast cells	6 (17.6)	5 (23.8)	0.284	1 (3.0)	4 (19.0)	0.06
Vasculitis	5 (14.7)	4 (19.0)	0.079	2 (6.1)	2 (9.5)	0.841
Necrosis	2 (5.9)	2 (9.5)	0.068	2 (6.1)	10 (47.6)	0.004*
Panniculitis	10 (62.5)δ	0 (0)	<0.001*	6(75.0%)ε	0 (0)	0.001*

δ; n=16 ε; n= 8, * statistical test significant at *P=0.05*

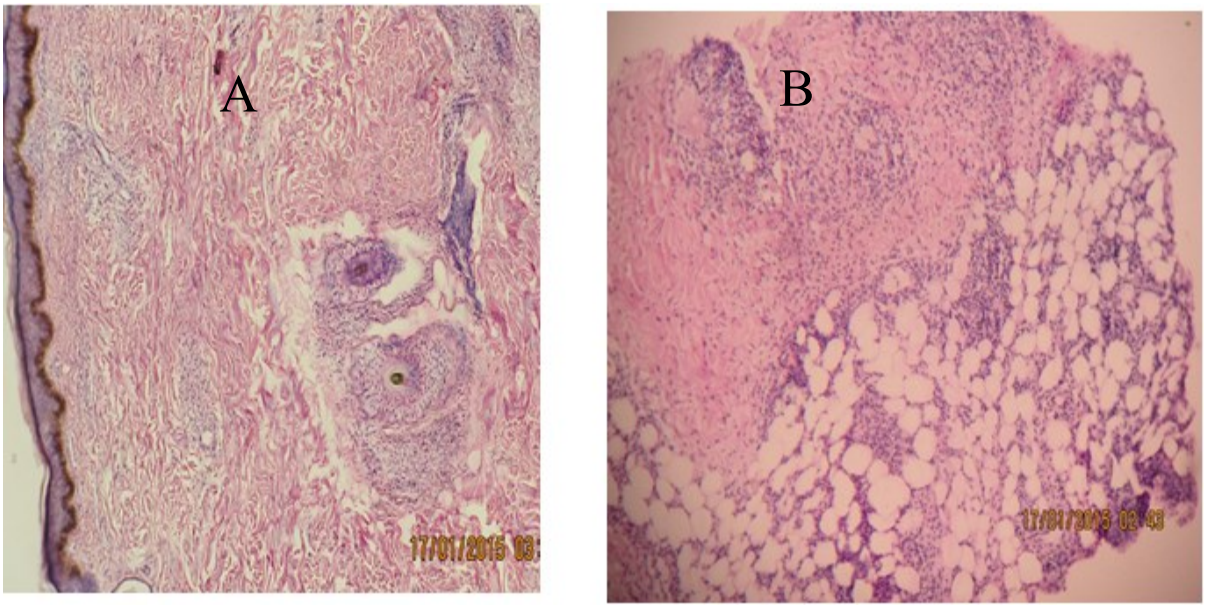


Figure 6.1.5. H &E stained skin biopsies: A. Histopathology of LL lesion without reaction: reticular dermal infiltration of lymphocytes, flat epidermis and foamy histiocytes. B. Histopathology of EN lesion: flat granular PMN infiltration with perivascular lymphocytic infiltration and lobar panniculitis. H & E staining x40

1. 3. Lipid profile at enrolment

Lipid metabolism during infection is thought to be mediated by cytokines which modulate the immune and inflammatory responses. It has been reported that changes in lipid and lipoprotein metabolism are part of the acute phase response of leprosy reactions. In our study, we analysed the serum lipid profile in patients with ENL and LL controls at enrolment.

The levels of serum triglycerides were low (99.95 ± 6.046 SE mg/dl) in patients with ENL reactions compared to LL patient controls (158.7 ± 7.394 SE mg/dl) ($P<0.0001$). Serum total cholesterol (TC) was significantly lower in patients with ENL reactions (137.3 ± 7.049 mg/dl) compared to LL patient controls (175.8 ± 8.805 mg/dl) at enrolment ($P<0.005$). Patients with ENL reactions had 19.20 ± 1.565mg/dl mean total serum high-density lipoproteins (HDL) which were significantly lower than the serum HDL of LL patient controls (29.76 ± 1.491mg/dl) before treatment ($P<0.0001$). Similarly, mean serum low density lipoprotein (LDL) was also considerably lower in

patients with ENL reactions (40.29 ± 3.596 mg/dl) than in LL patient controls (110.8 ± 3.380mg/dl) (P<0.0001) at enrolment (Figure 6.1.6).

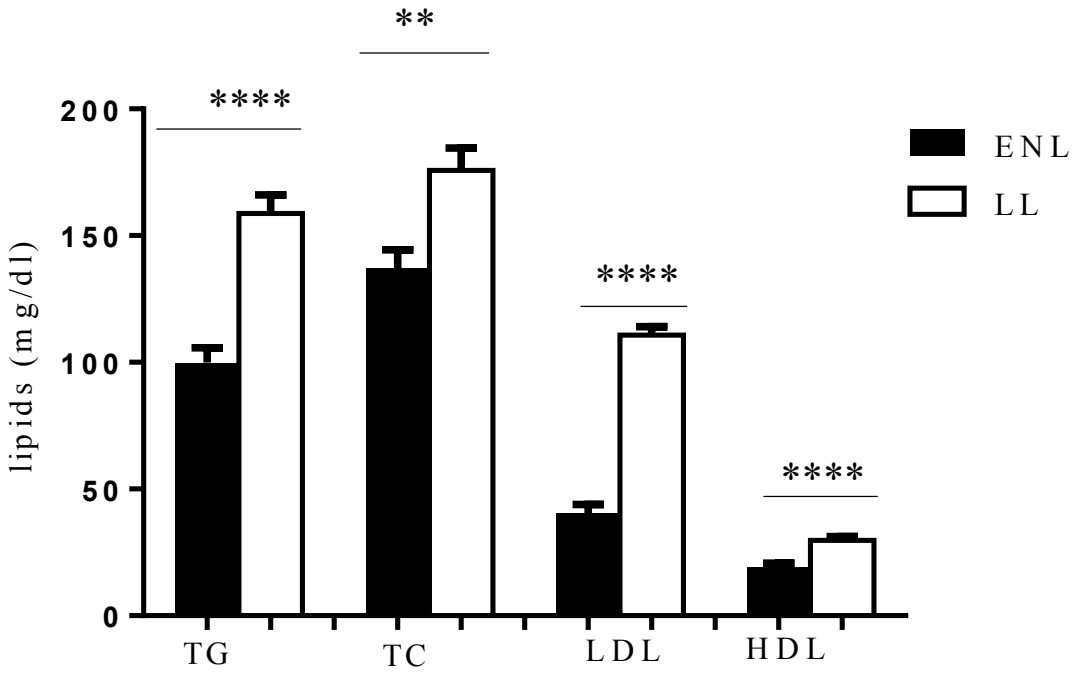


Figure 6.1.6. Lipid profiles of patients with ENL cases (n=46) and LL controls (n=31) at enrolment (mean ± standard error of the mean). TG = Triglycerides, TC= total cholesterol, LDL= low density lipoprotein, HDL= high density lipoprotein. ** P≤ 0.005; **** P≤0.0001.

SECTION 2: REGULATORY T-CELLS

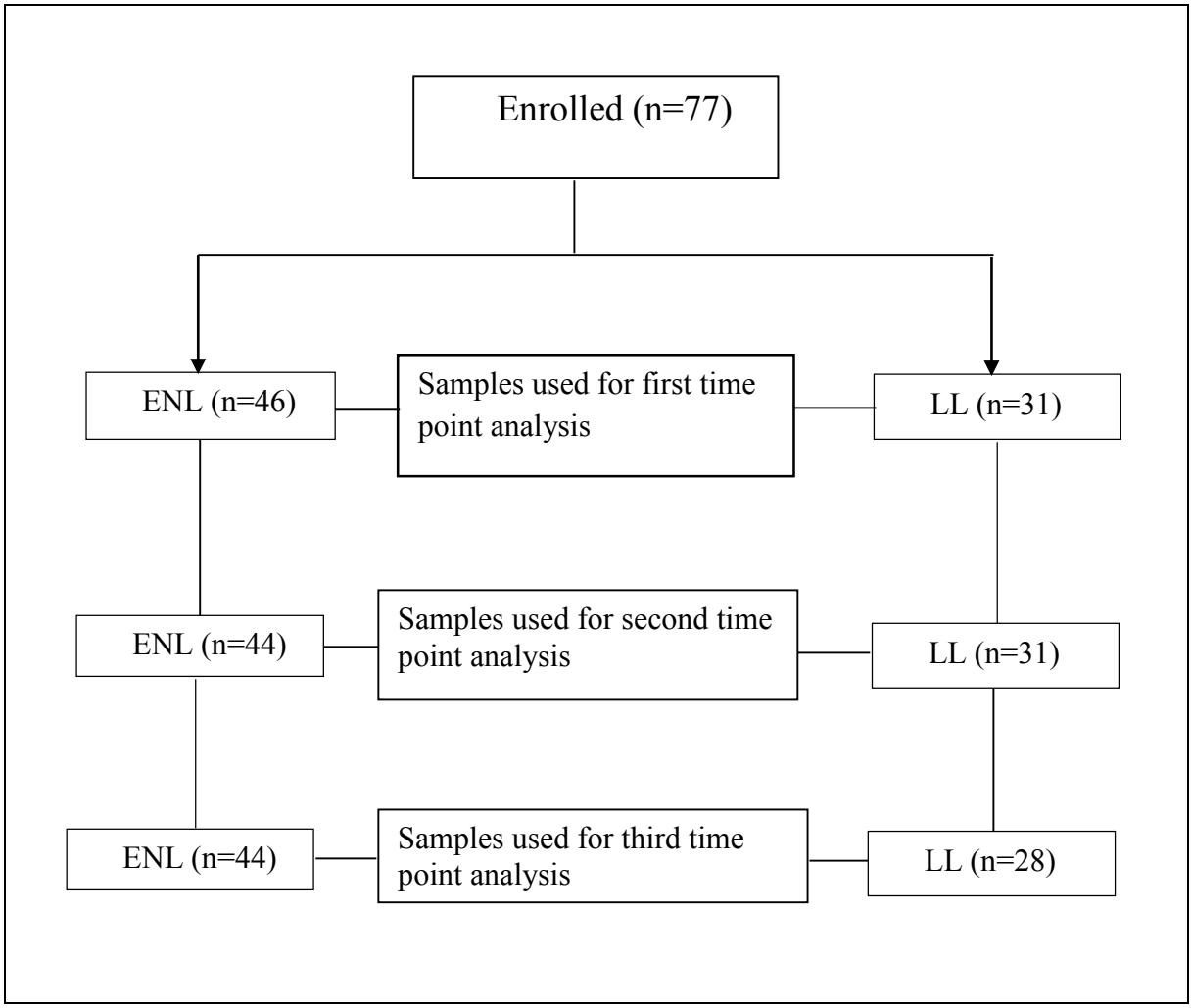


Figure 6. 2.1. Flowchart showing number of samples used for flow cytometry assays

2.1. Optimization

For the analysis of T and B cells on 8 colour BD FACSCanto II was used. Unstained cells, fluorescent minus one (FMO) staining, compensation controls and live/dead stains were used for quality control. Antibody titration was performed to determine the minimum amount of antibody required to achieve antigen binding saturation as described before. The photomultiplier tubes (PMTs) voltage were adjusted for all fluorochromes used in this study as follows: SSC-319, FSC- 434, FITC-580, PE- 483, PerCP-Cy5.5- 487, PE-Cy7- 620, APC- 620, eFluoro780-620, APC450 (Pacific Blue)-508 and live/dead -594. Fluorescent compensation was done during each date acquisition and automatically linked to the global worksheet (Figure 6.2.2).

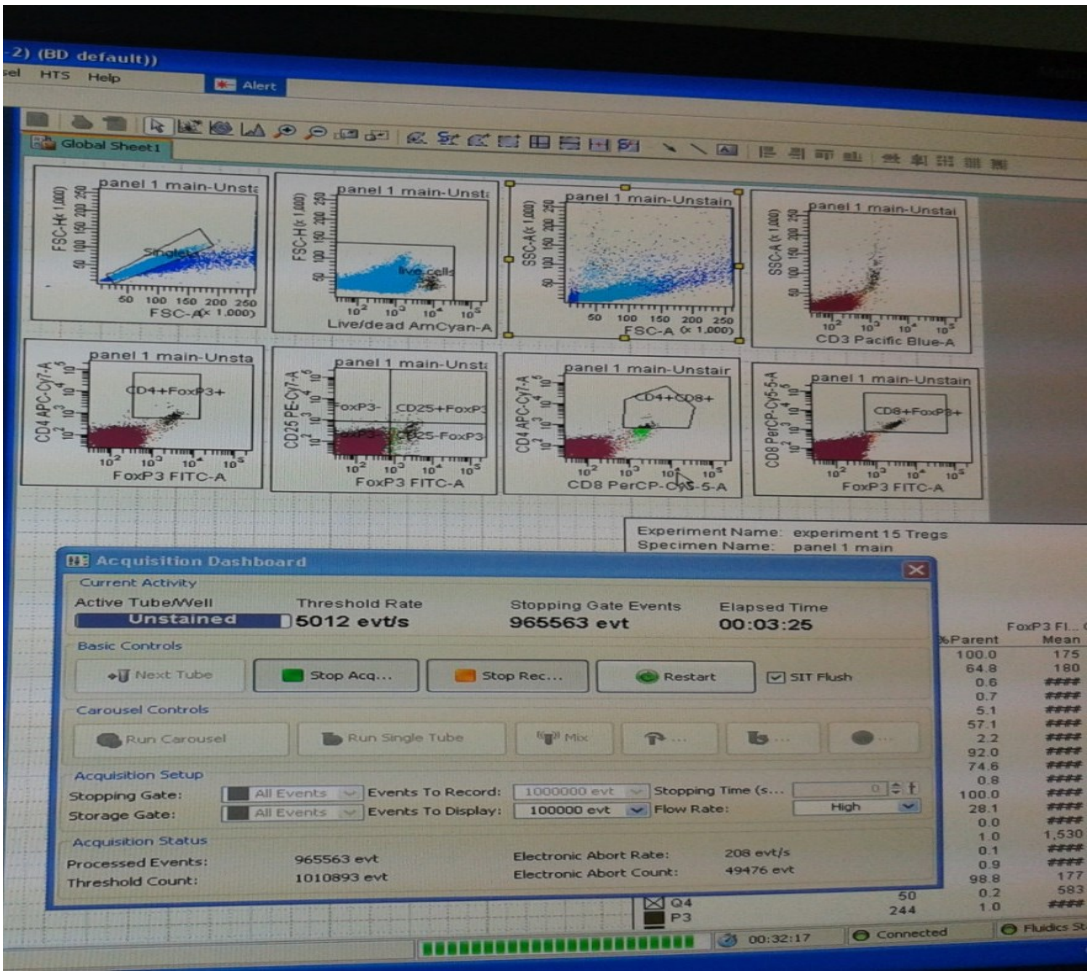


Figure 6.2.2. Data acquisition on cytometer window

2.1. Regulatory T cells in patients with ENL and LL controls

2.1.1. Introduction

To investigate the relative percentage of T-lymphocytes before, during and after prednisolone treatment of patients with ENL, venous blood samples were collected at each time point from patients with ENL and LL controls. Peripheral blood mononuclear cells (PBMCs) were isolated and stained with antibody conjugated fluorochromes. Stained cells were acquired on a flow cytometer and the percentage of each subset of T-lymphocytes were obtained by gating according to their forward (FSC) and sidescatter (SSC) properties.

The median percentage of T-lymphocytes in patients with ENL and LL controls were compared using Mann-Whitney (U) test. We used non-parametric statistical analysis since the distribution of flow cytometry data follows non-Gaussian distribution. Fluorescent intensity increases logarithmically and the use of an arithmetic mean for such data leads to statistical bias. The median is considered as the most robust test statistic for a non-Gaussian distribution since it is less influenced by skewed values or outliers. The effect size was calculated using Hodges-Lehmann statistic (ΔHL) estimates of the median of the population. Hodges-Lehmann estimates the differences between the populations of the paired random variables drawn respectively from the populations and it is less sensitive to the outliers and to the heterogeneity of the data (Lunneborg, 2014). Values for Hodges-Lehmann (ΔHL) and the 95% confidence interval for the median are given in tables (Appendices 5-7).

2.1.2. $CD4^+$ and $CD8^+$ T- lymphocytes

The median percentage of $CD4^+$ and $CD8^+$ T-cells in patients with ENL and LL controls are indicated by box-and-whisker plots (Figure 6.2.3). Patients with ENL reactions had a significantly higher percentage of median expression for $CD4^+$T-cells (61.3%) compared to LL patient controls (49.1%) at enrolment ($P < 0.0001$; ΔHL= 12.8 %). During prednisolone treatment of patients with ENL reaction, the median percentage for $CD4^+$ T cells decreased to 54.2% while that of LL patient controls increased to 61.4% and the difference was statistically significant ($P \leq 0.05$). Although the percentage median expression for $CD4^+$ T-cells had slightly increased to 58.1% in patients with ENL reactions after treatment, it remained significantly lower than in

LL patient controls (64.7%) and the difference was statistically significant ($P \leq 0.005$) (Figure 6.2.3).

On the other hand, patients with ENL had a significantly lower median percentage of $CD8^+$ T-cells (27.0%) before treatment compared to LL patient controls (35.7%) and the difference was statistically significant ($P<0.0001$; $\Delta HL= 8.2\%$). Interestingly, while patients with ENL are on treatment, unlike $CD4^+$ T-cells the median percentage of $CD8^+$ T-cells increased to 34.4% and it was higher than that of LL patient controls (28.3%) ($P<0.001$). After treatment, the corresponding values did not show significant changes in patients with ENL (33.5%) and LL controls (27.2%) (Figure 6.2.3).

The ratio of $CD4^+$ to $CD8^+$ T-cell was also determined before, during and after treatment of patients with ENL to see the trends in the balance of the two main T-cell types in patients with ENL compared to the LL controls during the follow-up period. The $CD4^+/CD8^+$ T-cell ratio was higher (2.3: 1) in patients with ENL compared to LL patient controls (1.4:1) before treatment ($P<0.001$; $\Delta HL= 0.09$). However, a significantly lower $CD4^+/CD8^+$ T-cell ratio was seen in patients with ENL (1.7: 1) compared to LL patient controls (2.25:1) during treatment ($P\leq 0.001$). After treatment, the ratio of $CD4^+/CD8^+$ T- cell was 2.14: 1 and 1.8:1 in patients with ENL and LL controls respectively and the difference was not statistically significant ($P>0.05$) (Figure 6.2.3). Hence, this result indicates that patients with ENL reactions and non-reactional LL controls not only show significant differences in the percentage of $CD4^+$ and $CD8^+$ T-cells but also in $CD4^+$ / $CD8^+$ T-cell ratio. Thus, patients with ENL had higher median percentages of $CD4^+$ and $CD4^+/CD8^+$ T-cell than LL patient controls before treatment.

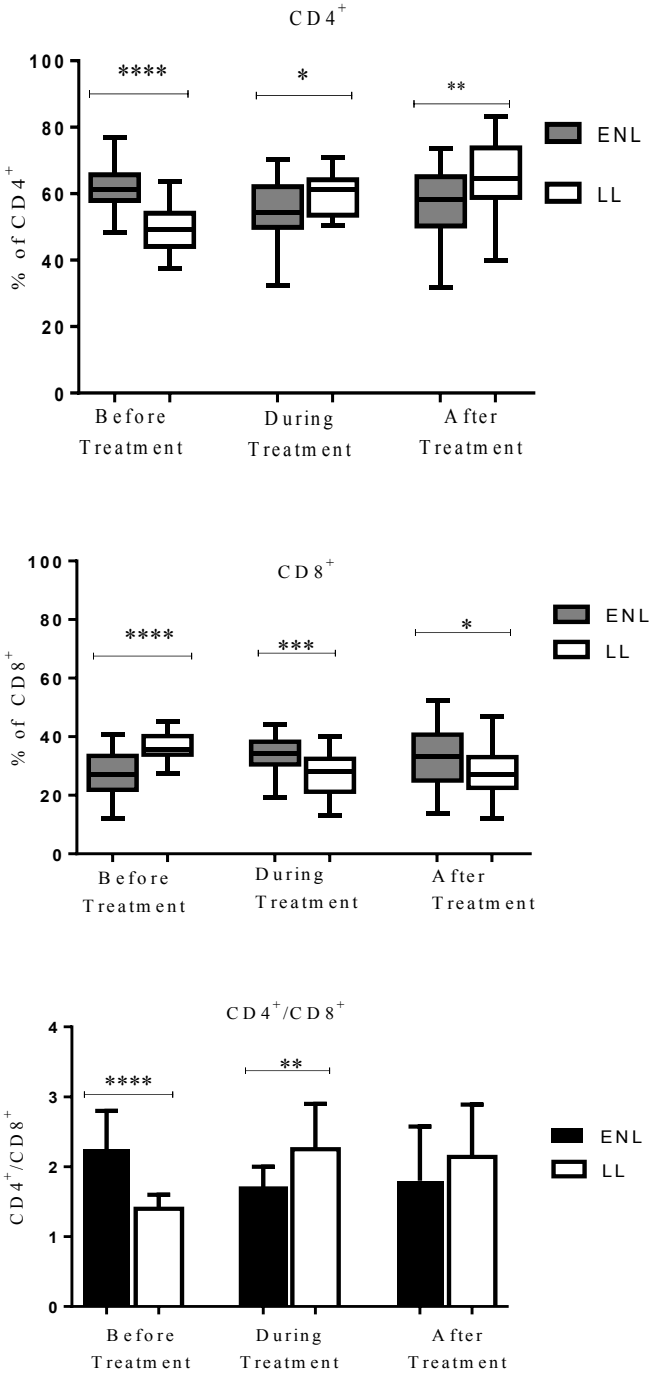


Figure 6.2.3. T-lymphocytes in patients with ENL cases and LL controls: median percentage of $CD4^+$T-cells, $CD8^+$ T-cells and the median percentage ratio of $CD4^+$ to $CD8^+$ T- cells in the PBMCs of patients with ENL reaction and LL controls before, during and after treatment of ENL cases. ENL: n (before) =46, n (during) = n (after) = 44; LL: n (before) = n (during) = 31, n (after) =28. Statistical test: Mann-Whitney unpaired test (U). * *P≤ 0.05*; ** *P≤ 0. 0.005*; *** *P≤0.001*; **** *P<0.0001*. Bar graphs show median ± interquartile range

2.1.3. Expression of CD25 by T-lymphocytes

Initially, it was thought that cells expressing CD25, the alpha chain of the IL-2 receptor, had a regulatory function. However, it was found that CD25 is also expressed on conventionally activated T cells that are not regulatory type and not all peripheral regulatory T-cells express CD25. Nevertheless, many authors still use CD25 as a regulatory marker particularly in mycobacterial research including leprosy research.

We investigated the median percentage of CD25 expression by $CD4^+$ and $CD8^+$ T-cells in the PBMCs from patients with ENL and LL controls before, during and after treatment of patients with ENL. The median percentage with interquartile range was used to plot a bar graph indicating the relative difference of the percentage of CD25 cells expression by $CD4^+$ and $CD8^+$ T-cells in these patient groups (Figure 6.2.4).

The median percentage of $CD4^+$ T-cells expressing $CD25^+$ T-cells in the PBMCs of ENL and LL patient controls were almost identical (8.9% and 8.8% respectively) before treatment. About 2.6% of $CD8^+$ T-cells had expressed CD25 in the PBMCs from patients with ENL and a slightly higher proportion of $CD8^+$ T-cells (3.2%) was expressed CD25 in the PBMCs from LL patient controls before treatment ($P \leq 0.05$). The expression of CD25 on both $CD4^+$ and $CD8^+$ T- cells during and after treatment did not change in both patient groups (Figure 2.4). Hence, these results indicate that the expression of CD25 by $CD4^+$ T-cells is similar in patients with ENL and LL controls and it does not discriminate ENL from LL.

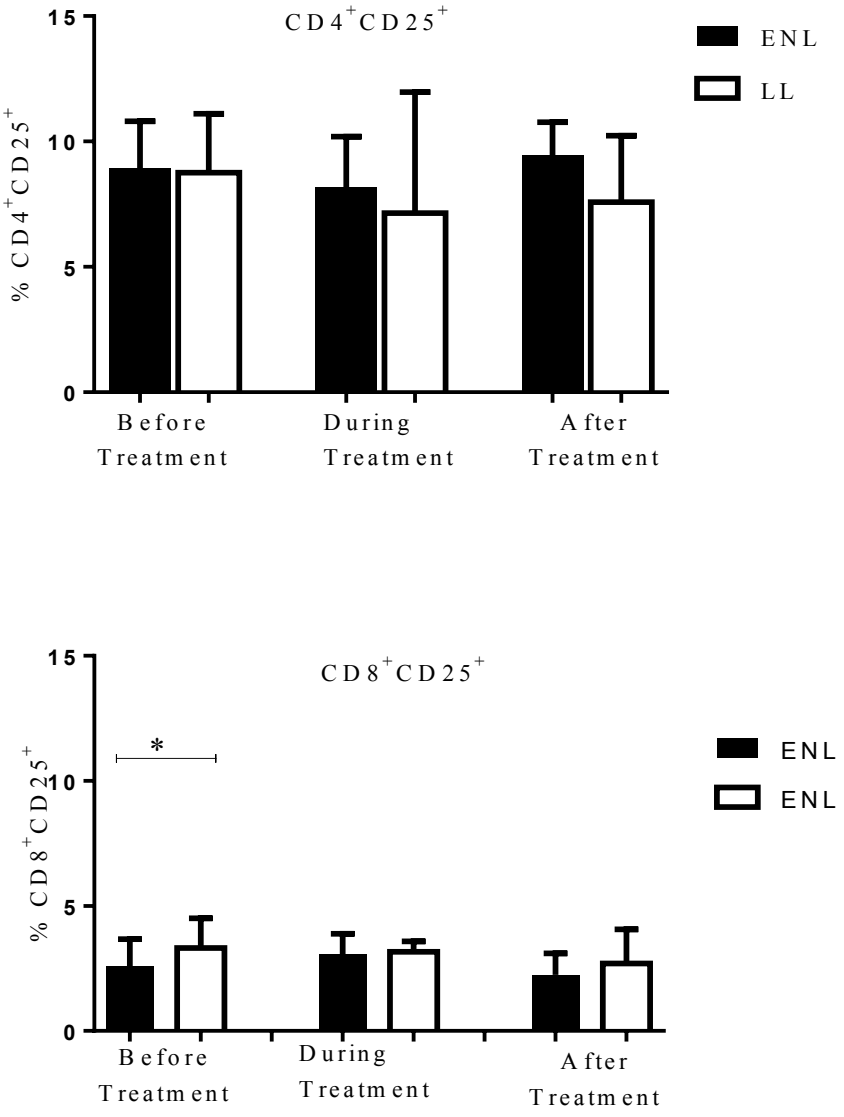


Figure 6.2.4. The median percentage of CD25 expression on $CD4^+$ and $CD8^+$ T-cells in the PBMCs of patients with ENL and LL patient controls before, during and after treatment. ENL: n (before) =46, n (during) = n (after) = 44; LL: n (before) = n (during) = 31, n (after) =28. Statistical test: Mann-Whitney unpaired test (U). * $P \leq 0.05$; ** $P \leq 0.\ 0.005$. **Bar** graphs show median ± interquartile range.

2.1.4. FoxP3 Expression in $CD4^+$ and $CD8^+$ T-lymphocytes

FoxP3 (forkhead box P3), is considered as the key transcription factor controlling the differentiation and function of regulatory T-cells (Cretney et al., 2013). FoxP3 has been shown to have important implications in various diseases. Studies have shown that the overexpression of FoxP3 in T-lymphocytes leads to progression of leprosy by downregulating T-cell activation (Kumar et al., 2014b).

The expression of FoxP3 in $CD4^+$ and $CD8^+$ T-cells were investigated in the PBMCs from patients with ENL and LL controls before, during and after prednisolone treatment of patients with ENL by flow cytometry. The median percentage of FoxP3-expressing $CD4^+$ T-cells in the PBMCs of patients with ENL was lower (2.1%) compared to the median percentage expressed in the PBMCs of LL patient controls (5.1%) before treatment ($P<0.0001$). During treatment, the frequency of FoxP3-expressing $CD4^+$T- cells slightly increased to 3.5% in patients with ENL and decreased by half from 5.1% to 2.6% in LL controls and the difference between the two groups was statistically significant ($p\leq0.05$). However, the frequency of FoxP3-experssing $CD4^+$ T-cells in the PBMCs from patients with ENL and LL controls did not show statistically significant differences after treatment of patients with ENL (Figure 6.2.5) implying the possible association of reduced percentage of $CD4^+$ $FoxP3^+$ T-cells with ENL reaction. This means that after ENL reaction recedes, the percentage of $CD4^+$ $FoxP3^+$T-cells in ENL cases and LL controls was not significantly different.

We also investigated the frequency of $CD8^+$ T-cells expressing FoxP3 in the peripheral blood of patients with ENL and compared to the corresponding LL patient controls before, during and after treatment. Although the median percentage of FoxP3-expressing $CD8^+$ T-cells in the PBMCs of patients with ENL was slightly lower (0.57%) compared to LL controls (0.71%) before treatment, the difference was not statistically significant ($P>0.05$). During treatment, patients with ENL had a lower frequency of $CD8^+FoxP3^+$ T-cells (0.49%) compared to LL patient controls (1.17%) ($P\leq0.05$). After treatment, the frequency of FoxP3 expression in $CD8^+$ T-cells in patients with ENL and LL controls was found to be similar ($P \geq0.05$) (Figure 6.2.5).

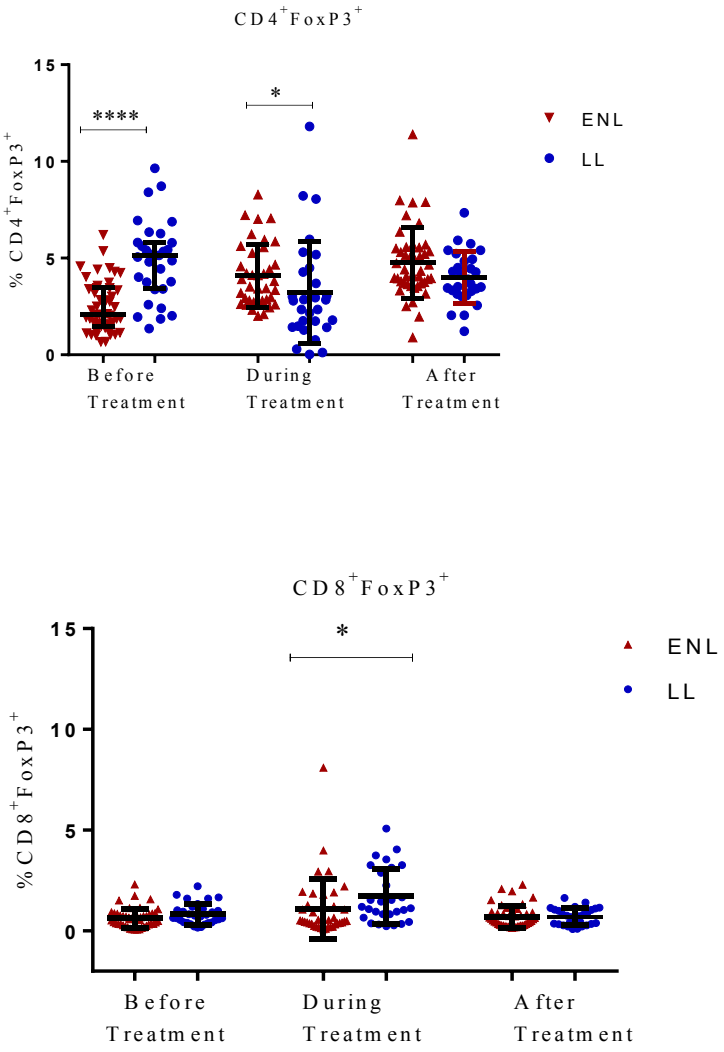


Figure 6.2.5. Median percentage of FoxP3 expression in $CD4^+$ and $CD8^+$ T-cells in the PBMCs of patients with ENL and LL controls before during and after prednisolone treatment of patients with ENL. ENL: n (before) =46, n (during) = n (after) = 44; LL: n (before) = n (during) = 31, n (after) =28. Statistical test: Mann-Whitney unpaired test (U). * $P \leq 0.05$; **** $P<0.0001$. **Error bars** show median ± interquartile range.

2.1.5. Expression of $CD25^+$ $FoxP3^+$ double positive T-lymphocytes

The frequency of $CD25^+FoxP3^+$ expression in $CD4^+$ and $CD8^+$ T-lymphocytes were measured in the PBMCs of patients with ENL and LL controls before, during and after treatment. About 1.8% of $CD4^+$ T-cells were expressed $CD25^+FoxP3^+$ in the PBMCs of patients with ENL which was significantly lower than the proportion of $CD25^+FoxP3^+$ cells expressed by $CD4^+$ T-cells (3.8%) in the PBMCs of LL patient controls ($P<0.0001$). During treatment, the median percentage of $CD4^+CD25^+FoxP3^+$ T-cells in the PBMCs of patients with ENL was increased to 2.6% while it was decreased from 3.8% to 2.5% in LL patient controls. After treatment, the percentage of $CD4^+CD25^+FoxP3^+$ T-cells in the PBMCs of patients with ENL was further increased to 3.3% while it was decreased to 2.2% in patients with LL controls and the difference was statistically significant ($P\leq0.001$) (Figure 6.2.6).

A small proportion of $CD8^+$ T- cells in the PBMCs of patients with ENL and LL controls expressed $CD25^+FoxP3^+$. The percentage of $CD8^+CD25^+FoxP3^+$ T- cells in the PBMCs of patients with ENL and LL controls was 0.4% and 0.6% respectively before treatment and slightly decreased in both groups (0.28% in patients with ENL and 0.52% in LL controls) during treatment. After treatment, these figures were slightly increased in both groups. The frequency of $CD8^+CD25^+FoxP3^+$ T- cells expression in the PBMCs of patients with ENL and LL controls was not significantly different before, during and after treatment (Figure 6.2.6). Hence, it appears that $CD4^+CD25^+FoxP3^+$ T- cells are more importantly associated with ENL reaction than the $CD8^+CD25^+FoxP3^+$ T- cells in patients with ENL.

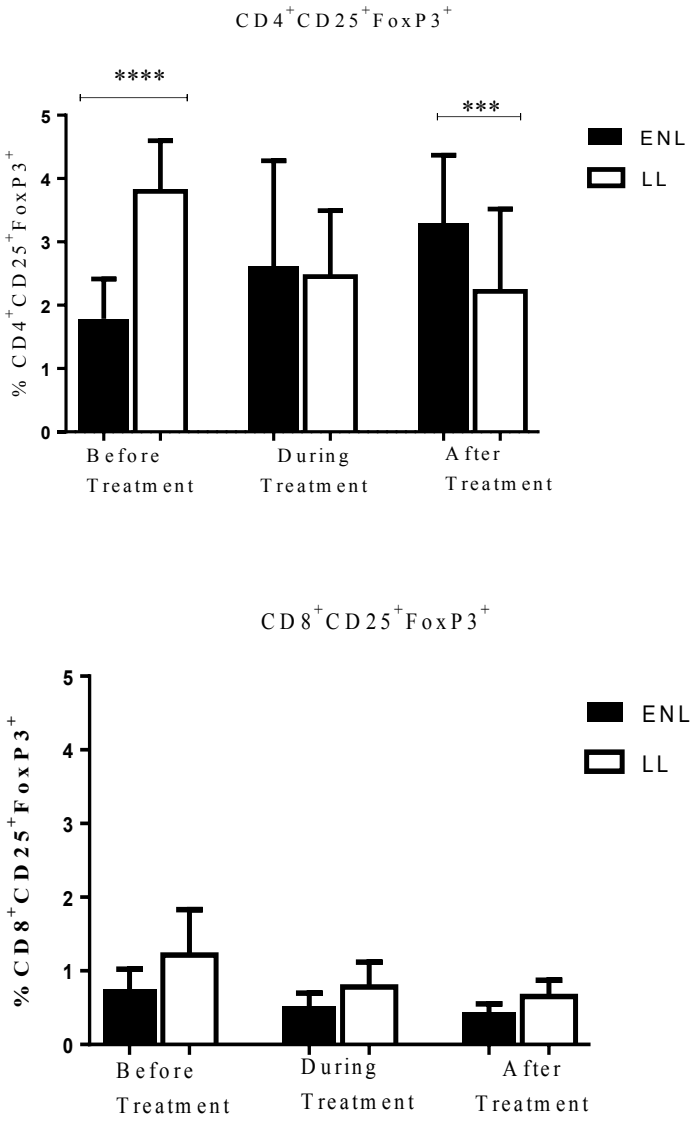


Figure 6.2.6. Median percentage of $CD4^+$ and $CD8^+$ T-cells expressing $CD25^+$ $FoxP3^+$ double positives in the PBMCs of patients with ENL reaction and LL controls before during and after prednisolone treatment of patients with ENL. ENL: n (before) =46, n (during) = n (after) = 44; LL: n (before) = n (during) = 31, n (after) =28. Statistical test: Mann-Whitney unpaired test (U). *** *P≤0.001;* **** *P<0.0001*. **Bar** graphs show median ± interquartile range.

2.1.6. $CD4^+$ and $CD8^+$ regulatory T- cells

Although several markers have been proposed for identification of Tregs in humans, none of them found to be an absolute marker and the search for perfect Treg markers continues. In leprosy, $CD4^+CD25^{hi}$ and $CD4^+CD25^+FoxP3^+$ have been used as Treg markers by several researchers. However, a consensus on the thresholds of CD25 expression needed to define Tregs within the CD25 high population is difficult to attain and variations in FoxP3 expression within CD25 high T-cells have been shown in patients as well as healthy individuals. Some studies have shown that the low expression of CD127 (an Interleukin-7 receptor subunit alpha), in conjunction of CD25 expression is one of the best strategies to identify a highly enriched $FoxP3^+$ population that encompasses the majority of $FoxP3^+$ T-cells. Based on these assumptions, we used $CD4^+CD25^+FoxP3^+$ $CD127^{-/lo}$ and $CD8^+CD25^+FoxP3^+$ $CD127^{-/lo}$ as $CD4^+$ and $CD8^+$ Treg markers respectively to investigate the relative proportion of regulatory T-cells in patients with ENL and LL controls.

The median percentage expression of $CD4^+$ regulatory T- cells was significantly lower (1.67%) in the PBMCs of patients with ENL compared to LL patient controls (3.79%) before treatment ($P \leq 0.0001$; $\Delta HL = 1.93\%$). During treatment, a significant difference was not observed with regard to the percentage of $CD4^+$ Tregs in the PBMCs of patients with ENL and LL controls. However, the percentage of $CD4^+$ Tregs in the PBMCs of patients with ENL was almost doubled (from 1.67% to 3.21%) while it was significantly decreased from 3.79% to 2.43% in LL patient controls during treatment (Figure 6.2.7 and Appendix 5). Hence, it appears that treatment of patients with ENL reactions with prednisolone increases the expression of $CD4^+$ Tregs and thus suppress the immune hyperactivation to resolve the inflammatory process in these patients. After treatment, 3.2% of $CD4^+$ T-cells expressed Tregs in patients with ENL while only 2.5% of $CD4^+$ T-cells expressed Tregs in patients with LL controls ($P \leq 0.005$) (Figure 6.2.7).

The median percentage of $CD8^+$ Tregs was slightly less in the PBMCs of patients with ENL (0.37%) compared to LL patient controls (0.54%) but it was not statistically significant ($P \geq 0.05$). During treatment, the median percentage of $CD8^+$ Tregs was slightly decreased in both groups (0.23% in patients with ENL and 0.42% in LL patient controls) ($P \geq 0.05$). The median percentage of $CD8^+$ Tregs after treatment of patients

with ENL and LL controls was 0.34% and 0.47% respectively (Figure 6.2.6). Hence, it appears that prednisolone treatment does not affect the percentage of $CD8^+$ Tregs unlike $CD4^+$ Tregs in ENL patients.

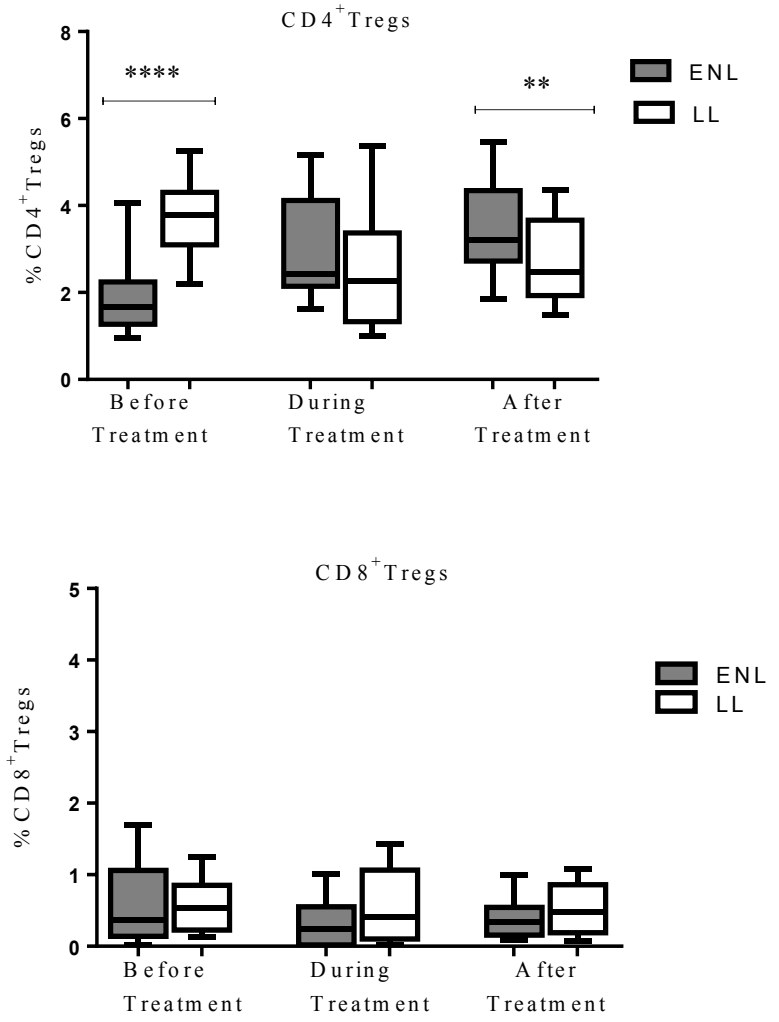


Figure 6.2.7. Median percentage of $CD4^+$ and $CD8^+$ regulatory T- cells ($CD4^+CD25^+FoxP3^+CD127^{-/lo}$ and $CD8^+CD25^+FoxP3^+CD127^{-/lo}$ respectively) in the PBMCs of patients with ENL reaction and LL controls before, during and after treatment. ENL: n (before) =46, n (during) = n (after) = 44; LL: n (before) = n (during) = 31, n (after) =28. Statistical test: Mann-Whitney unpaired test (U). ** P≤ 0. 0.005; **** P<0.0001. Box and whiskers show median ± interquartile range.

2.1.7. IL-17 producing cells

IL-17 has recently been drawn much attention in host defence and inflammatory diseases. While $CD4^+$ and $CD8^+$ T-cells are important sources of this cytokine, recent data have suggested that the γδ T cells and a number of families of innate lymphoid cells also secrete IL-17. In this study, the percentage of IL-17 producing lymphocytes and T-cells has been analysed. The percentage of IL-17 producing $CD4^+$ and $CD8_+$ T-cells was further analysed in the PBMCs of patients with ENL and LL controls before, during and after prednisolone treatment of patients with ENL.

Patients with ENL had a significantly higher median percentage of IL-17 producing lymphocytes (26.45%) than LL patient controls (20.6%) before treatment ($P \leq 0.05$; $\Delta HL=5.7$). Similarly, the proportion of IL-17 producing T-cells in the PBMCs of patients with ENL was considerably higher (23.1%) than in LL patient controls (18.4%) before treatment and the difference was statistically significant ($P \leq 0.05$; $\Delta HL=4.5$). In this study, it was found that while patients with ENL had a higher percentage of IL-17 producing $CD4^+$ T-cells than LL patient controls before treatment, the percentage of IL-17 producing $CD8^+$ T-cells was not significantly different in these patient groups. During and after treatment of patients with ENL, none of these cells showed a significant difference in patients with ENL and LL patient controls (Figure 6.2.8).

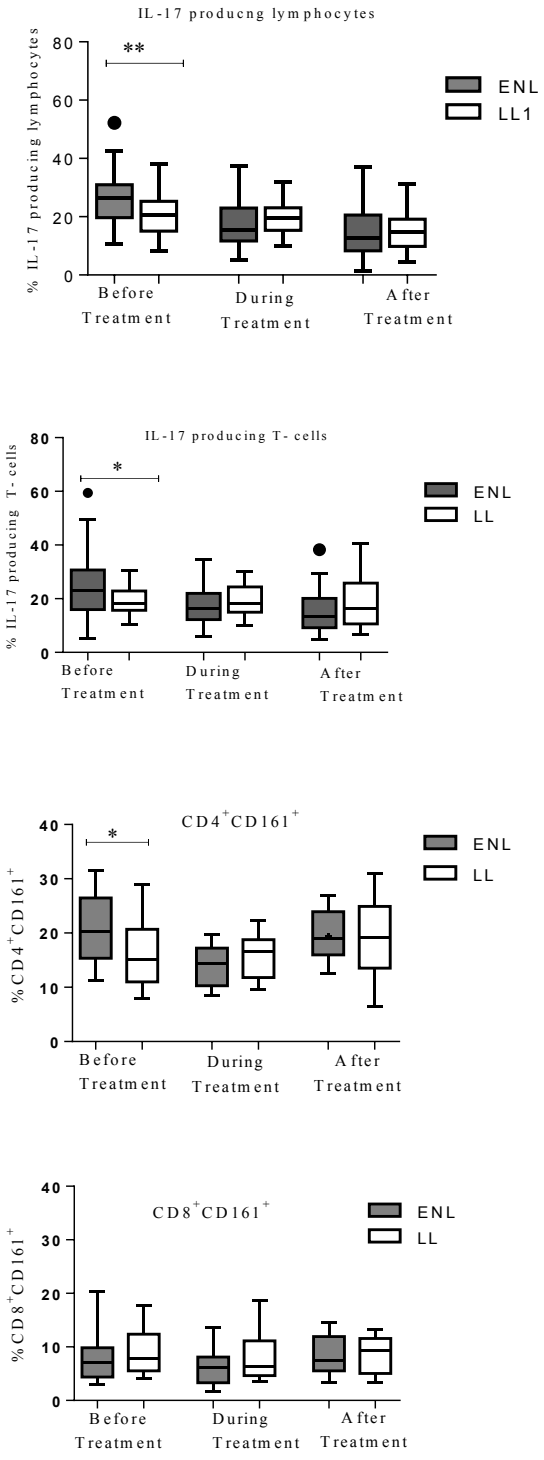


Figure 6.2.8. IL-17 Producing cells in the PBMCs of patients with ENL and LL controls before, during and after treatment. ENL: n (before) =46, n (during) = n (after) = 44; LL: n (before) = n (during) = 31, n (after) =28. Statistical test: Mann-Whitney unpaired test (U). *$P \leq 0.05$; ** $P \leq 0.\ 0.005$. Box and whiskers show median ± interquartile range

Table 6.2.1. Summary table showing trends of T-cell regulation before and after treatment of patients with ENL and LL controls.

T-cell sub population (%)	Before treatment		During treatment		After treatment	
	ENL	LL	ENL	LL	ENL	LL
$CD4^+$	↑	↓	↓	↑	↓	↑
$CD8^+$	↓	↑	↑	↓	↑	↓
$CD4^+/CD8^+$ *	↑	↓	↓	↑	–	–
$CD4^+CD25^+$	–	–	–	–	–	–
$CD8^+CD25^+$	↓	↑	–	–	–	–
$CD4^+FoxP3^+$	↓	↑	↑	↓	–	–
$CD8^+FoxP3^+$	–	–	↓	↑	–	–
$CD4^+CD25^+FoxP3^+$	↓	↑	–	–	↑	↓
$CD8^+CD25^+FoxP3^+$	–	–	–	–	–	–
$CD4^+$ Tregs	↑	↓	–	–	↑	↓
$CD8^+$ Tregs	–	–	–	–	–	–
IL-17^+ lymphocytes	↑	↓	–	–	–	–
IL-17^+T-cells	↑	↓	–	–	–	–
$CD4^+$IL-17^+	↑	↓	–	–	–	–
$CD8^+$IL-17^+	–	–	–	–	–	–

*ratio (without unit). ↑= increased, ↓= decreased, – = no change

2.2. Regulatory T- cells in individual patients with ENL before and after treatment

T-cell subsets in the PBMCs of patients with ENL before, during and after prednisolone treatment was analysed to see the trend of each T-cell subset over treatment time. Blood samples were obtained from each patient 3 times (before, during and after treatment) and used for T-cells immunophenotyping by flow cytometry.

2.2.1. $CD4^+$ and $CD8^+$ T- lymphocytes

The median percentage of $CD4^+$ and $CD8^+$ T-lymphocytes in the PBMCs of patients with ENL at each time point were determined. The median percentage of $CD4^+$ T-cells was significantly higher (61.3%) before treatment than during treatment (54.2%) ($P \leq 0.001$; $\Delta HL=6.8\%$). Similarly, the median percentage of $CD4^+$ T-cells in the PBMCs of patients with ENL was lower (57.3%) after prednisolone treatment than before treatment ($P \leq 0.05$; $\Delta HL = 4.6\%$). However, the median percentage of $CD4^+$ T-cells in these patients was not significantly different during and after treatment ($P > 0.05$) (Figure 6.2.9).

Unlike $CD4^+$ T-cells, patients with ENL reactions had significantly lower frequency of $CD8^+$ T-cells (37.0%) before treatment than during treatment (34.4%) ($P \leq 0.0001$; $\Delta HL = 6.8\%$). After treatment, the median percentage of $CD8^+$ T-cells remained high (34.3%) compared with before treatment ($P \leq 0.005$) (Figure 6.2.9). This finding shows that while prednisolone treatment decreased the median percentage of $CD4^+$ T-cells, it increased the median percentage of $CD8^+$ T-cells in patients with ENL reactions.

The median percentage ratio of $CD4^+$ to $CD8^+$ T-cells before, during and after treatment of patients with ENL was compared to see the trend of $CD4^+$ to $CD8^+$ T-cells ratio in these individual patients. The $CD4^+$ to $CD8^+$ T-cells ratio before treatment was significantly higher (2.23:1) than during treatment (1.8: 1) ($P < 0.0001$; $\Delta HL=0.6$) and after treatment (1.7:1) ($P \leq 0.005$; $\Delta H=0.5$) in these patients (Figure 6.2.9, Appendix 5).

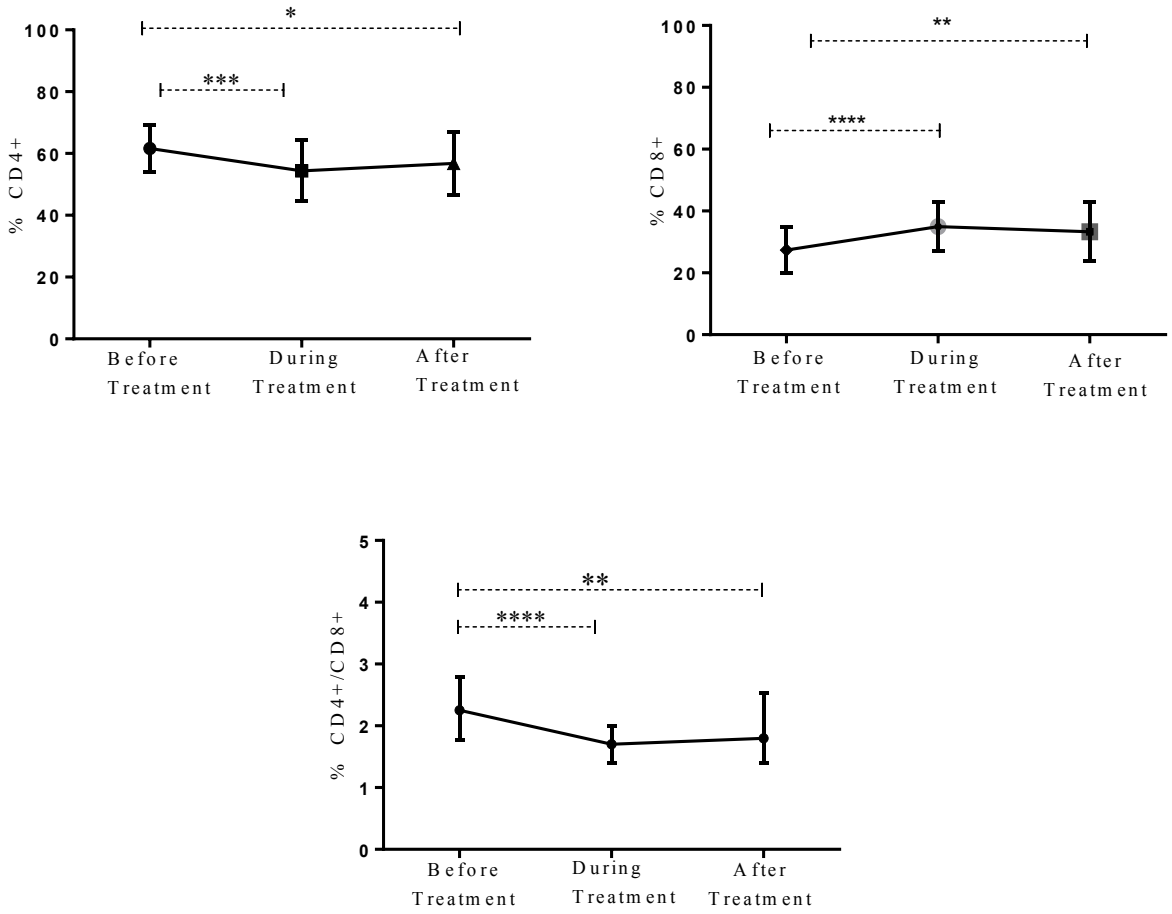


Figure 6.2.9. Median percentage of $CD4^+$T-cells, $CD8^+$ T-cells and the median percentage ratio of $CD4^+$ to $CD8^+$ T- cells in the PBMCs of patients with ENL reactions before, during and after treatment with prednisolone. n (before) = (during) = n (after) = 44; LL: n (before) = n (during) = (after) =28. Statistical test: Wilcoxon matched-pairs test. * *P≤0. 0.05;* ** *P≤0. 0.005;* *** *P≤0.001;* **** *P<0.0001*. **Error bars** show median ± interquartile range.

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2.2.2. Expression of CD25 and FoxP3 in T-lymphocytes

The expression of CD25 and FoxP3 by $CD4^+$ and $CD8^+$ T- cells in the PBMCs of patients with ENL before, during and after prednisolone treatment was investigated to see if there was any significant differential expression of CD25 and FoxP3 on T-lymphocytes at each time point of treatment status.

The median percentage expression of CD25 in $CD4^+$ T-cells before and during treatment was 8.9% and 8.2% respectively. After treatment with prednisolone, about 9.5% of $CD4^+$ T-cells expressed CD25. The expression of CD25 by $CD4^+$ T-cells before, during and after treatment was not statistically significantly different ($P > 0.05$) implying that prednisolone may not affect the expression of CD25 in $CD4^+$ T-cells (Figure 2.10). Similarly, the expression of CD25 in $CD8^+$ T-cells during and after prednisolone treatment of patients with ENL did not change ($P > 0.05$). About 2.6% of $CD8^+$ T-cells was found positive for CD25 staining before treatment. The median percentage of $CD8^+CD25^+$ T- cells during and after treatment was 3.2% and 2.2% respectively ($P > 0.05$) (Figure 6.2.10).

The median percentage of $CD4^+FoxP3^+$ T-cells was significantly lower (2.1%) than the median percentage obtained during treatment (3.5%) ($P<0.0001$; $\Delta HL = 1.4\%$) and after treatment (4.4%) ($P \leq 0.0001$; $\Delta HL = 2.2$). The expression of FoxP3 in $CD4^+$ T-cells was doubled after prednisolone treatment of patients with ENL indicating the association of prednisolone and FoxP3 expression unlike the CD25 expression in the PBMCs in these patients (Figure 6.2.10). Interestingly, the expression of FoxP3 in $CD8^+$ T-cells was not significantly different before (0.59%), during (0.49%) and after (0.50%) treatment of these patients. Hence, it appears that prednisolone treatment does not affect the expression of FoxP3 in $CD8^+$ T-cells unlike in $CD4^+$T-cells.

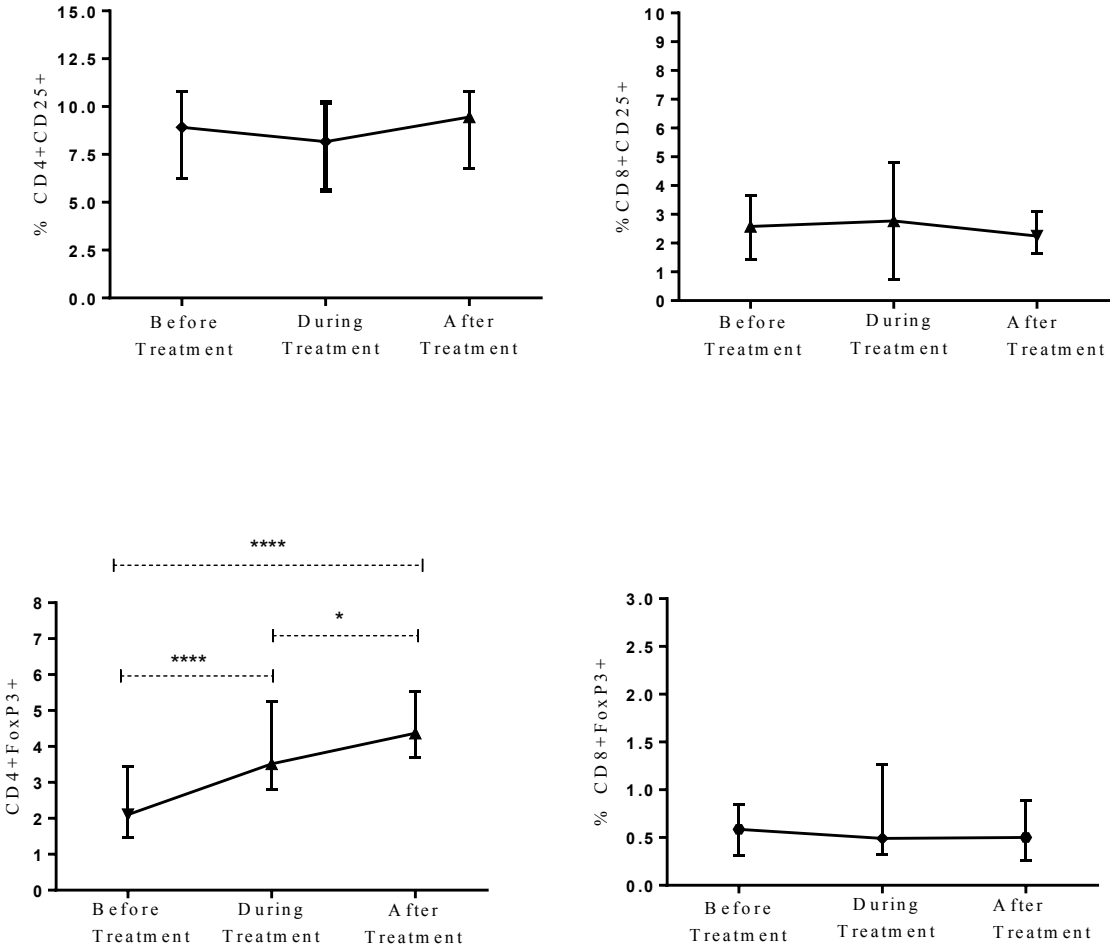


Figure 6.2.10. Median percentage of $CD4^+$ and $CD8^+$ T-cells expressing CD25 and FoxP3 in the PBMCs of patients with ENL before, during and after treatment with prednisolone. n (before) = (during) = n (after) = 44; LL: n (before) = n (during) = (after) =28. Statistical test: Wilcoxon matched-pairs test. * *P≤ 0.05*; **** *P <0.0001*. Error bars show median ± interquartile range.

2.2.3. CD25 FoxP3 double positive T-lymphocytes

The expression of double positive ($CD25^+FoxP3^+$) by $CD4^+$ and $CD8^+$ T-cells were investigated in the peripheral blood mononuclear cells of patients with ENL before, during and after treatment. The median percentage of $CD4^+CD25^+FoxP3^+$ T-cells in the PBMCs of patients with ENL was found to be significantly low before treatment compared with the median percentage obtained during treatment (2.6%; $P<0.0001$; $\Delta HL=1.04\%$) and after treatment (3.3%; $P<0.0001$; $\Delta HL=1.55\%$). On the other hand, the median percentage of $CD8^+CD25^+FoxP3^+$ T-cells was not significantly different before, during and after treatment (Figure 6.2.11).

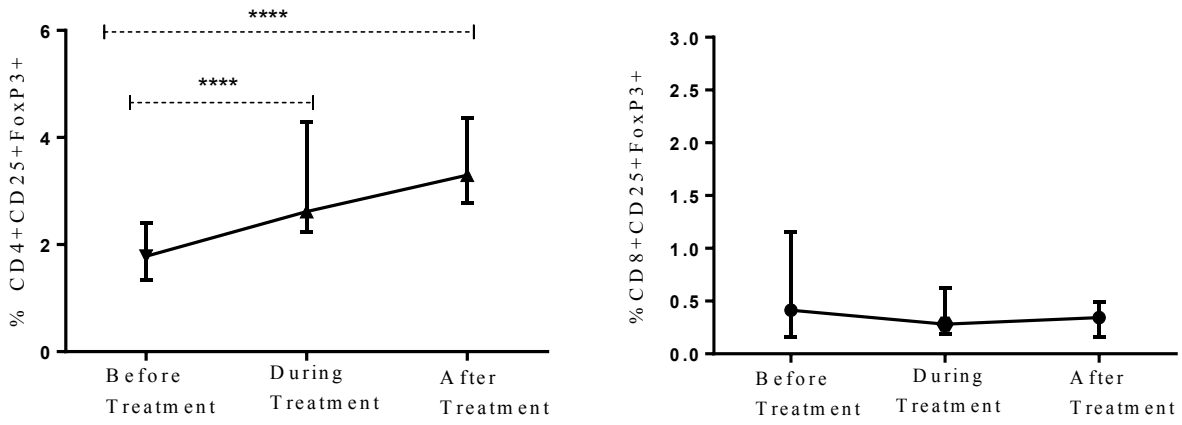


Figure 6.2.11. Median percentage of $CD25^+FoxP3^+$ expression by $CD4^+$ and $CD8^+$ T-cells in the PBMCs of patients with ENL before during and after treatment with prednisolone. n (before) = (during) = n (after) = 44; LL: n (before) = n (during) = (after) =28. Statistical test: Wilcoxon matched-pairs test. **** *P <0.0001*. Error bars show median ± interquartile range.

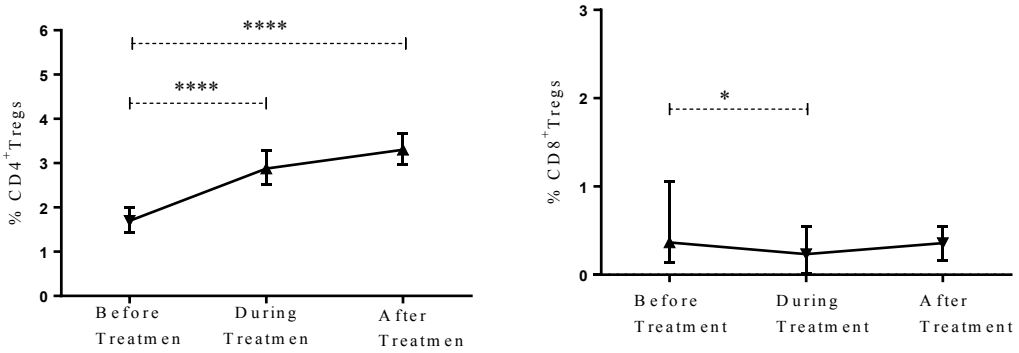
2.2.4. $CD4^+$ and $CD8^+$ regulatory T-cells

Regulatory T-cells expression in $CD4^+$ and $CD8^+$ T- cells were investigated before and after prednisone treatment of patients with ENL. The median percentage expression of $CD4^+$ regulatory T- cells was significantly lower (1.67%) in the PBMCs of patients with ENL reactions before treatment compared with the median percentage of Tregs expressed in these cells during treatment (2.5%) ($P<0.0001$; $\Delta HL=1.0\%$). After treatment, the median percentage of $CD4^+$ Tregs increased to 3.20% compared with the percentage (1. 67%) obtained before treatment ($P<0.0001$; $\Delta HL =1.6$ %) (Figure 6.2. 12). Thus, this result suggests that ENL reaction is associated with decreased percentage of $CD4^+$ Treg cells.

In contrast to $CD4^+$ Tregs, the percentage of $CD8^+$ Tregs was not significantly different before and after prednisolone treatment of patients with ENL. The median percentage of $CD8^+$ Tregs before, during and after treatment was 0.37%, 0.23% and 0.36% respectively ($P>0.05$) (Figure 6.2.12).

The median percentage of $CD4^+$ and $CD8^+$ Tregs were highly variable when individual patients are considered. While, in the majority of patients with ENL, the median percentage of $CD4^+$Tregs increased after treatment, in few cases it was decreased after treatment. On the other hand, the median percentage of $CD8^+$ Tregs remained stable in the majority of patients with ENL cases but in few instances, it was either increased or decreased (Figure 6.2.12).

A



B

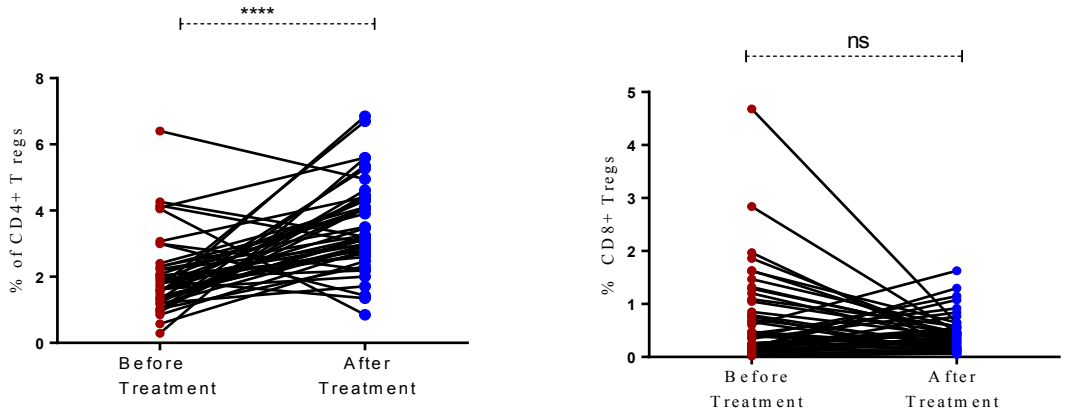


Figure 6.2.12. The median percentage of Tregs expression in $CD4^+$ and $CD8^+$ T-cells. (A) The median percentage of $CD4^+$ and $CD8^+$ Tregs in the PBMCs of patients with ENL before during and after treatment with prednisolone. (B) Trends in the percentage of individual $CD4^+$ and $CD8^+$ Tregs in the PBMCs of patients with ENL reaction before and after treatment with prednisolone. n (before) = (during) = n (after) = 44; LL: n (before) = n (during) = (after) =28. Statistical test: Wilcoxon matched-pairs test. *$P \leq 0.05$; **** $P<0.0001$; ns= non-significant. Error bars show median ± interquartile range.

2.2.5. IL-17 producing cells

The median percentage of IL-17 producing cells was compared in patients with ENL cases before, during and after treatment to see the trend of these cells during the follow-up period. The median percentage of IL-17 producing lymphocytes in the PBMCs of patients with ENL reaction was significantly higher before treatment (26.45%) than during treatment (15.4%) ($P<0.0001$, $\Delta HL=9.5\%$). After treatment, the median percentage of these IL-17 producing lymphocytes was further decreased to 12.7% and it was significantly lower than before treatment ($P<0.0001$, $\Delta HL=11.855\%$). Similarly, the proportion of IL-17 producing T-cells was substantially higher (23.1%) before treatment than during treatment (16.4%) and after treatment (13.2%) ($P<0.0001$) (Figure 6.2.13).

The median percentage of IL-17 producing $CD4^+$ and $CD8^+$ T-cells were also investigated in these patients before and after treatment to see the source of IL-17. It was found that the proportion of IL-17 producing $CD4^+$ T-cells was significantly decreased during treatment ($P< 0.0001$) but again increased after treatment. Unlike IL-17 producing lymphocytes, the median percentage of IL-17 producing $CD4^+$ T-cells was not significantly different before and after treatment showing that $CD4^+$ T-cells may not be the main source of IL-17 in these patients. IL-17 producing $CD8^+$ T-cells did not show any significant difference before, during and after treatment of patients with ENL reaction (Figure 6.2.13). Hence, prednisolone treatment of patients with ENL reactions does not affect IL-17 producing $CD8^+$T-cells but it could affect transiently IL-17 producing $CD4^+$T-cells.

In conclusion, ENL reaction was associated with decreased frequency of $CD4^+$ regulatory T-cells, increased percentage of $CD4^+FoxP3^+$ T-cells and increased $CD4^+/CD8^+$ T-cell ratio. On the other hand, $CD8^+$ regulatory T-cells, the expression level of CD25 by $CD4^+$ and $CD8^+$ T-cells and the expression of $CD8^+FoxP3^+$ T-cells were not associated with ENL reaction.

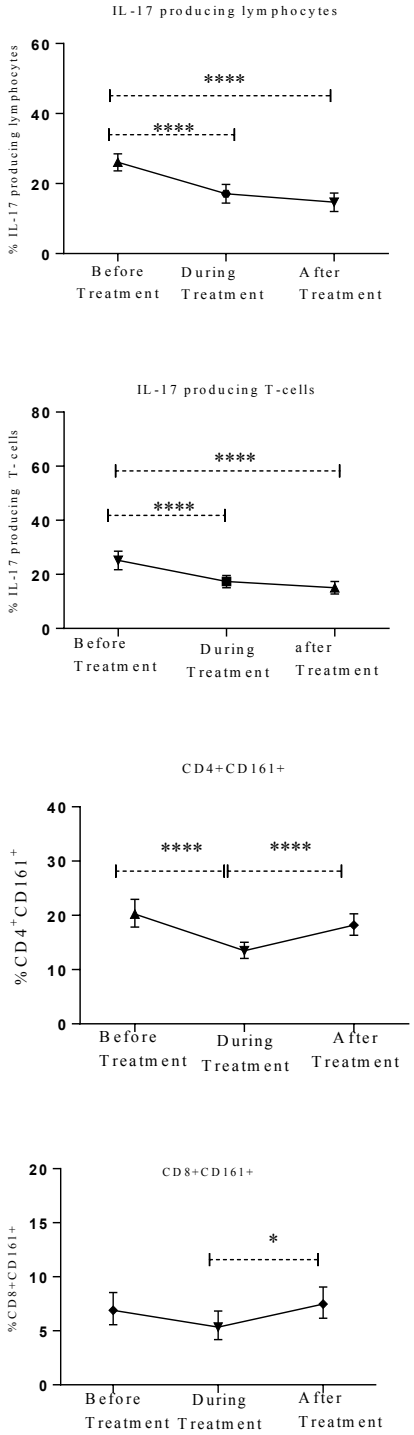


Figure 6.2.13. IL-17 Producing cells in the PBMCs of patients with ENL before, during and after treatment. n (before) = (during) = n (after) = 44; LL: n (before) = n (during) = (after) =28. Statistical test: Wilcoxon matched-pairs test. *$P \leq 0.05$; ****$P < 0.0001$. Error bars show median ± interquartile range.

Table 6.2.2. Summary table showing trends of T-cell regulation in patients with ENL before and after treatment.

T-cell sub population (%)	Before treatment	After treatment
$CD4^+$	↑	↓
$CD8^+$	↓	↑
$CD4^+/CD8^+$ *	↑	↓
$CD4^+CD25^+$	–	–
$CD8^+CD25^+$	–	–
$CD4^+FoxP3^+$	↓	↑
$CD8^+FoxP3^+$	–	–
$CD4^+CD25^+FoxP3^+$	↓	↑
$CD8^+CD25^+FoxP3^+$	–	–
$CD4^+$ Tregs	↓	↑
$CD8^+$ Tregs	–	–
IL-17^+ lymphocytes	↑	↓
IL-17^+T-cells	↑	↓
$CD4^+$IL-17^+	–	–
$CD8^+$IL-17^+	–	–

*ratio (without unit). ↑= increased, ↓= decreased, – = no change

SECTION 3: ACTIVATED AND MEMORY T-CELLS

3.1. Introduction

Memory is the hallmark of the acquired immune system. It results from the clonal expansion and differentiation of antigen-specific lymphocytes that ultimately persist for a lifetime. Memory lymphocytes confer immediate protection in peripheral tissues and mount recall responses to antigens in secondary lymphoid organs. In the T-cell system, these functions are carried out by distinct cell types. Protective memory is mediated by effector memory T-cells (T_{EM}) that migrate to inflamed peripheral tissues and display immediate effector function, whereas reactive memory is mediated by central memory T-cells (T_{CM}) that home to T-cell areas of secondary lymphoid organs. Central memory T-cells readily proliferate and differentiate to effector cells in response to antigenic stimulation. Memory T- cells are distinguished by the expression of CD45RO. Central memory T-cells express CD62L but effector memory T-cells do not express this ligand. While naïve T-cells are characterized by their expression of CD62L but not CD45RO, effector T-cells do not express CD62L and CD45RO**.**

In leprosy, only one study tried to identify memory T-cell subsets (Mitra et al., 1999a) although, the study did not use an experimental design that allowed to discriminate the identification of memory T cell subsets. The expression level of each memory T-cell subsets and naïve T- cells in the PBMCs of patients with ENL and LL controls before and after treatment was investigated.

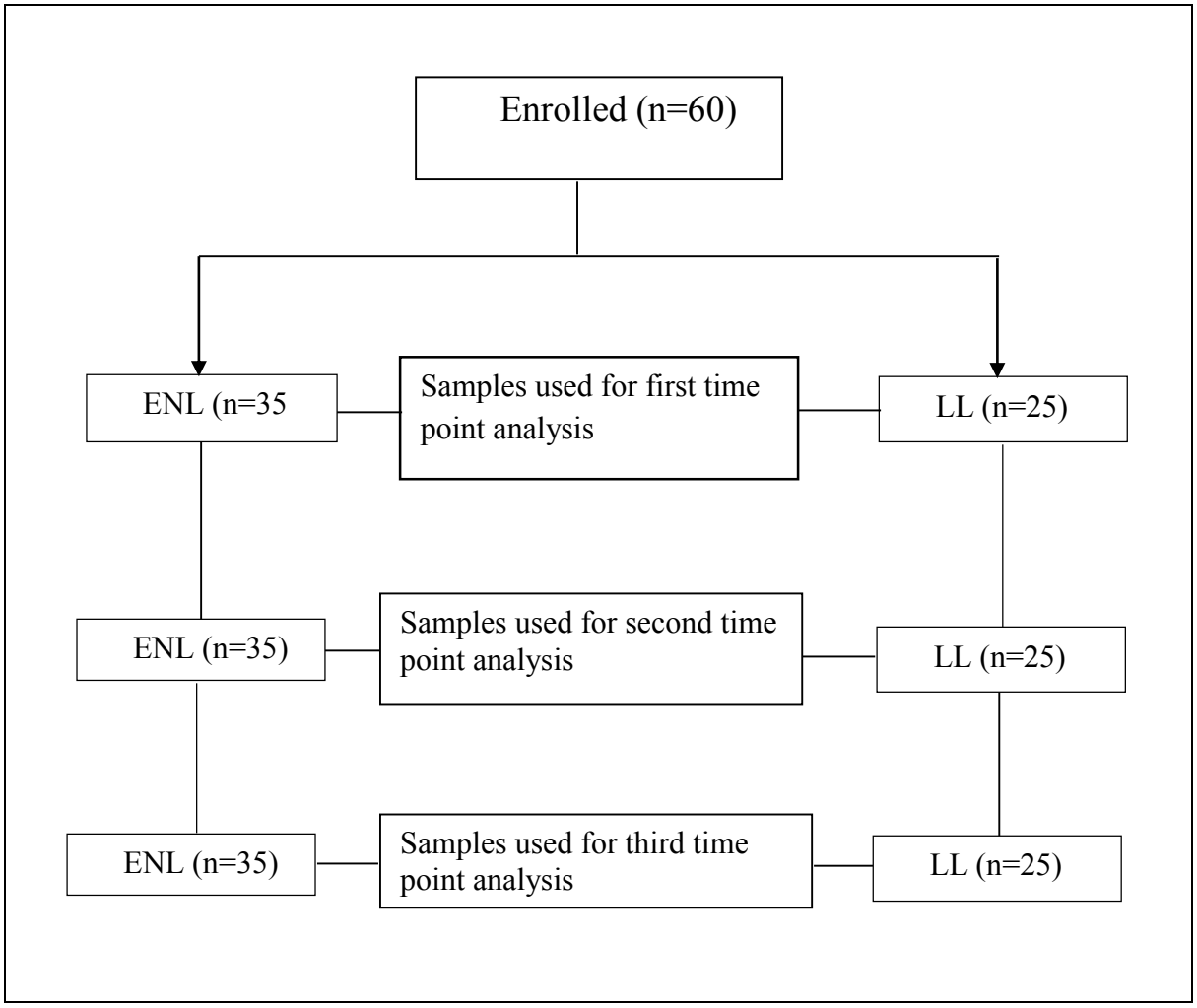


Figure 6. 3.1. Flowchart showing the number of samples used for memory T-cell assays

3.2. Activated and memory T-cells in patients with ENL and LL controls

3.2.1. Memory T-cells

About 40% of PBMCs from patients with ENL expressed $CD3^+$ memory T-cell ($CD3^+CD45RO^+$) compared to 28% of $CD3^+$ $CD45RO^+$ T-cells in LL patient controls before treatment, showing that a significantly higher proportion of $CD3^+$ T-cells in patients with ENL are antigen-experienced cells than in LL patient controls ($P \leq 0.005$; $\Delta HL = 10.5\%$). However, during treatment, the proportion of $CD3^+$ memory T-cells in patients with ENL was decreased to 29.2% whereas it remained almost at the same level in LL patient controls (29.3%) and the difference between the two groups was not statistically significant ($P>0.05$). Similarly, the percentage of $CD3^+$ memory T-cells after treatment of patients with ENL and LL controls were 31.2% and 32.7% respectively ($P>0.05$) (Figure 6.3.2).

More than 50% of $CD4^+$ T-cells were $CD45RO^+$ in patients with ENL which was significantly higher than in LL patient controls (30.5%) ($P < 0.0001$; $\Delta HL=20.3\%$) before treatment. During treatment, the proportion of antigen-experienced $CD4^+$ T-cells slightly decreased from 52.3% to 43.8% in patients with ENL while it was increased from 30.5% to 45.9% in LL patient controls but the difference was not statistically significant ($P> 0.05$). After treatment, the proportion of $CD4^+$ memory ($CD4^+CD45RO^+$) T-cells were 45.0% and 41.8% in patients with ENL and LL controls respectively ($P>0.05$) (Figure 6.3.2).

The median percentage of $CD8^+$ memory ($CD8^+CD45RO^+$) T-cells in patients with ENL (17.5%) and LL controls (15.2%) was not significantly different before treatment ($P>0.05$). The percentage of $CD8^+$ memory T- cells did not show a significant change during and after treatment (figure 6.3.2).

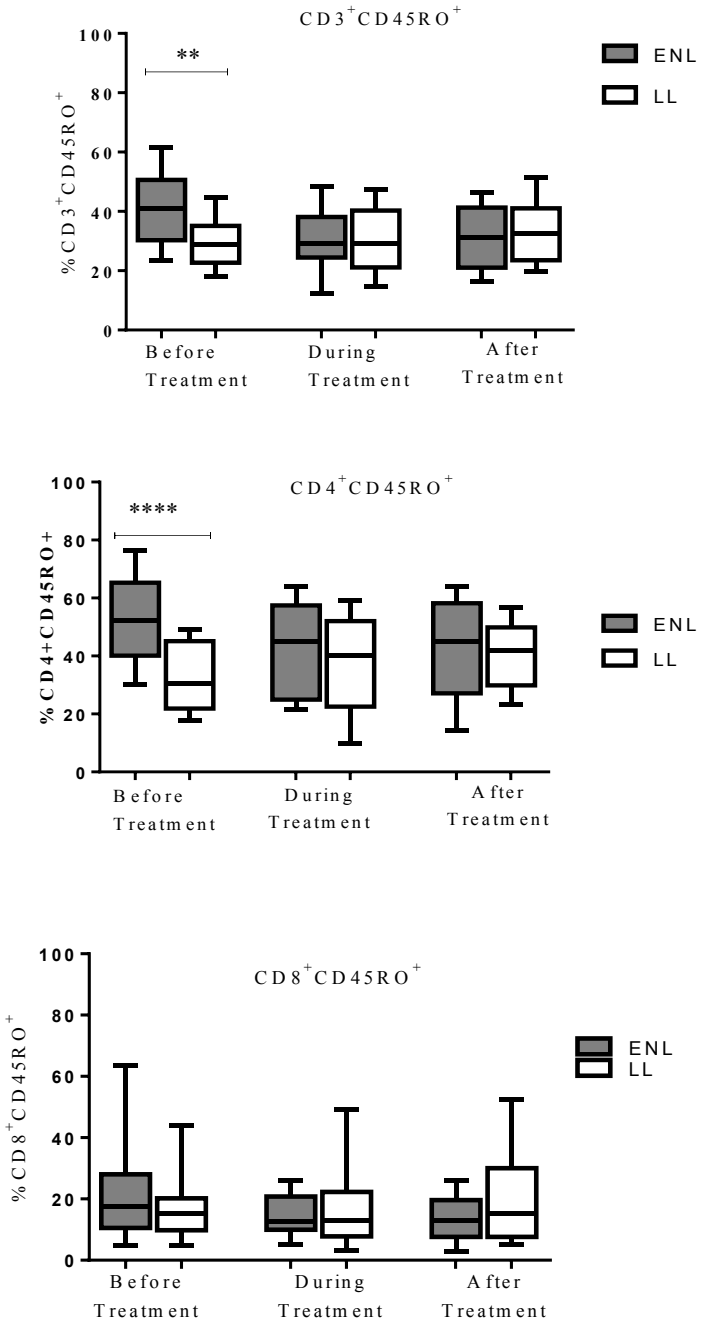


Figure 6.3.2. Median percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells expressing memory T-cells in the PBMCs of patients with ENL and LL controls before, during and after treatment. n (before) = (during) = n (after) = 35; LL: n (before) = n (during) = (after) =25. Statistical test: Mann-Whitney unpaired test (U). ** *P≤0.005*; **** *P<0.0001.*

3.2.2. Activated T- cells

The median percentage of activated T-cells in unstimulated PBMCs of patients with ENL reaction and LL controls was measured using CD62L as a T-cell activation marker. CD62L is a glycoprotein also known as L-selectin which serves a homing receptor for lymphocytes to enter secondary lymphoid tissues via high endothelial venules. During T- cell activation, CD62L is shed from the surface, resulting in $CD62L^-$ effector cells. Hence, effector memory T-lymphocytes do not express L-selectin, as they circulate in the periphery and have immediate effector functions upon encountering antigen.

A significant proportion of $CD3^+$ T-cells (59.2%) were activated in patients with ENL ($CD3^+CD62L^-$) in contrast to in LL patient controls (37.7%) before treatment ($P < 0.0001$; $\Delta HL = 22.4\%$). Interestingly, while the proportion of activated $CD3^+$ T-cells decreased to 47% in patients with ENL, it was increased to 49% in LL patient controls during treatment ($P > 0.05$) showing that prednisolone treatment of patients with ENL is associated with a decreased frequency of activated $CD3^+$ T-cells. After treatment, the proportion of $CD3^+$ T- cells further decreased to 33.9% in patients with ENL (Figure 6.3.3).

Similarly, the median percentage of activated $CD4^+$ T-cells was ($CD4^+CD62L^-$) significantly higher (50.7%) in PBMCs of patients with ENL than in LL controls (27.1%) before treatment ($P < 0.0001$; $\Delta HL = 19.1\%$). During treatment, the frequency of activated $CD4^+$ T-cells decreased by half to 29.7% in patients with ENL but increased to 38.1 % in LL patient controls yet the difference was not statistically significant ($P > 0.05$). After treatment, the proportion of activated $CD4^+$ T- cells in both groups remained almost at the same level with that of during treatment (Figure 6.3.3).

With regard to $CD8^+$ activated T- cells ($CD8^+CD62L^-$), while nearly two third (71.2%) was activated in patients with ENL, less than half (45.4%) was activated in LL patient controls ($P < 0.0001$; $\Delta HL = 27.9\%$). The median percentage of $CD8^+$ activated T- cells in patients with ENL cases (59.5%) and LL controls (49.7%) was not significantly different during treatment ($P > 0.05$). However, after treatment, the frequency of activated $CD8^+$ T-cells was significantly decreased to 34.5% in patients with ENL compared to 45.2% in LL patient controls ($P \leq 0.05$; $\Delta HL = 10.1\%$) (Figure 6.3.3).

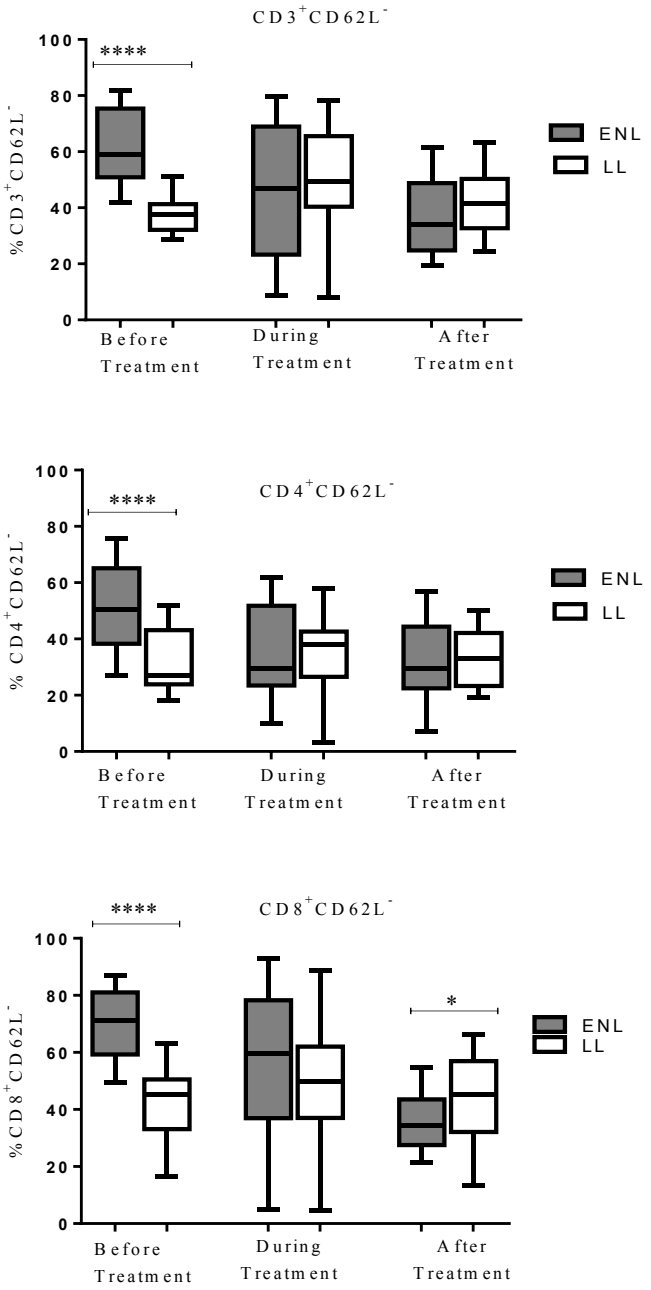


Figure 6.3.3. The median percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells expressing CD62L in unstimulated PBMCs of patients with ENL and LL controls before, during and after treatment. n (before) = (during) = n (after) = 35; LL: n (before) = n (during) = (after) =25. Statistical test: Mann-Whitney unpaired test (U). * $P \leq 0.05$; **** $P < 0.0001$. Box and whiskers show median ± interquartile range.

3.2.3. Effector memory T-cells

Effector memory T-cells (T_{EM}) are memory T-cells which have lost their CD62L expression while migrating to the tissue and progressively gain functionality with further differentiation to effector T- cells also called terminally differentiated T-cells(Seder et al., 2008). Measurement of effector memory T- cells is the most commonly used method to determine the extent of T-cell activation in a disease state. We measured the proportion of effector T- cells in unstimulated PBMCs from patients with ENL and LL controls before and after treatment to prove that the hypothesis that ENL is associated with T- cell activation.

The median percentage of $CD3^+$ effector memory T-cells ($CD3^+CD45RO^+$ $CD62L^-$) in the PBMCs of patients with ENL was 26.6% which was significantly higher than in LL patient controls (8.0%) before treatment ($P<0.0001$; $\Delta HL=18.3\%$). During treatment, the median percentage of $CD3^+T_{EM}$ was decreased to 16.5% in patients with ENL while it was appreciably increased to 13.0% in LL patient controls but the difference was not statistically significant ($P>0.05$). The percentage of $CD3^+$ T_{EM} was slightly lower in patients with ENL (7.6%) than in LL patient controls (10.4%) after treatment ($P\leq 0.05$; $\Delta HL= 3.5\%$) (Figure 6.3.4).

The median percentage of $CD4^+$ effector memory T- cells ($CD4^+CD45RO^+CD62L^-$) in the PBMCs of patients with ENL was nearly three times (24.6%) higher than in the PBMCs of LL patient controls (8.9%) before treatment ($P<0.0001$; $\Delta HL= 18.4\%$). During treatment, the proportion of $CD4^+$ T-cells expressing T_{EM} cells in patients with ENL was decreased to 11.4% while it was significantly increased to 17.6% in LL patient controls ($P\leq 0.05$; $\Delta HL= 5.8\%$). However, after treatment, the proportion of $CD4^+$ T-cells expressing T_{EM} in patients with ENL and LL controls were 9.8% and 13.1% respectively and the difference was not statistically significant between the two groups ($P>0.05$) (Figure 6.3.4).

Similarly, the median percentage expression level of effector memory T-cells in $CD8^+$ T-cells ($CD8^+CD45RO^+$ $CD62L^-$) was twice (16.5%) in patients with ENL compared to in LL patient controls (7.2%) before treatment ($P< 0.001$; $\Delta HL= 6.7\%$). However, during treatment, the percentage of $CD8^+$ T- cells expressing T_{EM} was reduced by more than half in patients with ENL (7.5%) and nearly remained at the same level in LL patient controls (8.3%) but the difference was not significantly different ($P> 0.05$).

After treatment, the median percentage of $CD8^+$ effector memory T-cells in patients with ENL and LL controls did not show a significant difference (Figure 6.3.4).

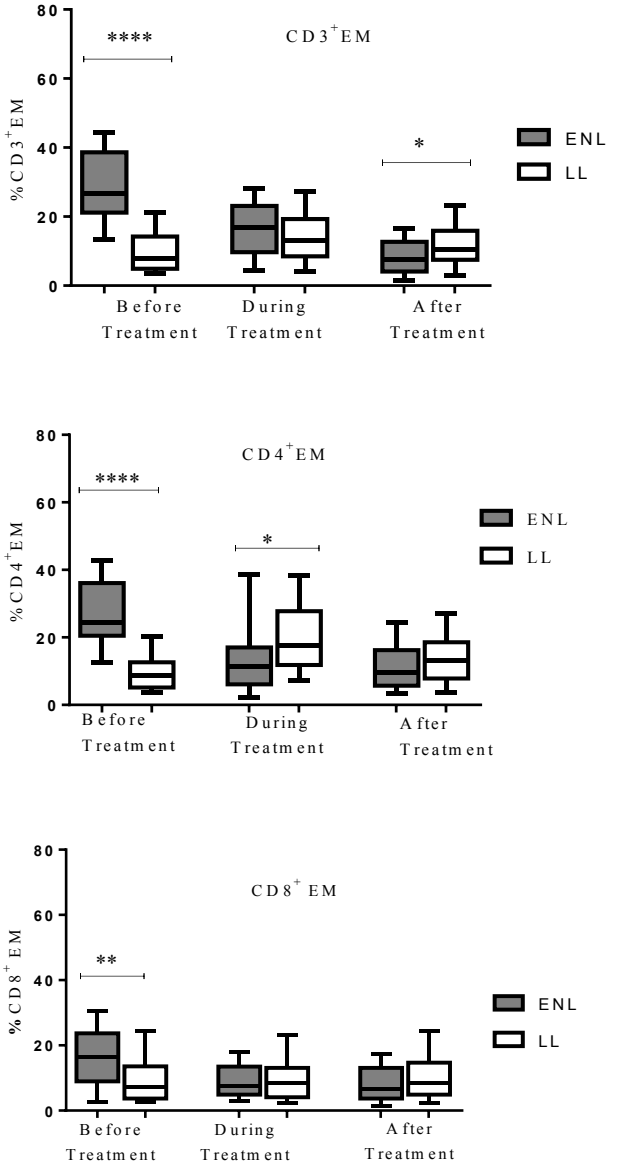


Figure 6.3.4. The median percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells expressing effector memory T-cells ($CD45RO^+CD62L^-$) in PBMCs of patients with ENL and LL controls before, during and after treatment. n (before) = (during) = n (after) = 35; LL: n (before) = n (during) = (after) =25. Statistical test: Mann-Whitney unpaired test (U). * $P \leq 0.05$; ** $P \leq 0.005$; **** $P < 0.0001$. Box and whiskers show median ± interquartile range.

3.2.4. Central Memory T-cells

Human central memory T-cells (T_{CM}) are $CD45RO^+$ memory cells that constitutively express CCR7 and CD62L. Upon proliferation, they differentiate to effector memory T- cells. The relative proportion of central memory T- cells expressing T- lymphocytes was measured in the PBMCs from patients with ENL and non-reactional LL controls before, during and after treatment to investigate the association of ENL reaction with T-cell activation.

The median percentages of T_{CM} expressing $CD3^+$ T-cells in patients with ENL and LL controls before, during and after treatment were not statistically significantly different ($P>0.05$). However, unlike $CD3^+T_{CM,}$ the proportion of $CD4^+$ T_{CM} was significantly higher in patients with ENL (23.5%) than in LL patient controls (14.6%) before treatment ($P\leq 0.005$; $\Delta HL= 8.13\%$). Although the median percentage of $CD4^+$ T_{CM} decreased from 23.5% to 13.7% in patients with ENL during treatment, a statistically significant difference was not arrived at compared to LL patient controls (18.1%) ($P>0.05$). Similarly, the median percentage of $CD4^+$ T_{CM} was not significantly different between the two groups after treatment (Figure 6.3.5).

Interestingly, the median percentage of $CD8^+$ T_{CM} was significantly lower in patients with ENL (1.2%) than in LL patient controls (3.5%) before treatment ($P \leq 0.0001$; $\Delta HL=2.3\%$). During treatment, the proportion of $CD8^+$ T_{CM} expressing cells in patients with ENL remained at a lower level (1.9%) than in LL patient controls (2.5%) but was not statically significantly different ($P>0.05$). After treatment, the percentage of $CD8^+T_{CM}$ was slightly higher in patients with ENL (2.7%) than in LL patient controls (2.0%) however the difference was not statistically significant ($P>0.05$) (Figure 6.3.5).

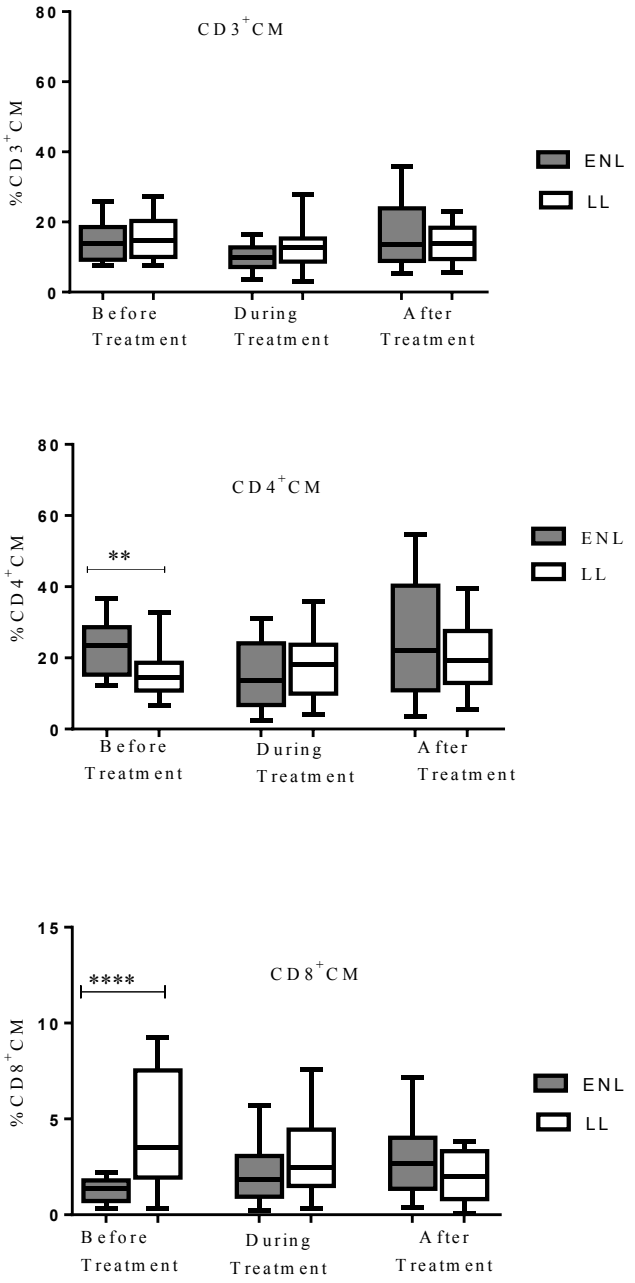


Figure 6.3.5. The median percentage of central memory T-cell expression in $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in PBMCs of patients with ENL and LL controls before, during and after treatment. n (before) = (during) = n (after) = 35; LL: n (before) = n (during) = (after) =25. Statistical test: Mann-Whitney unpaired test (U). ** $P \leq 0.005$; **** $P < 0.0001$. Box and whiskers show median ± interquartile range.

3.2.5. Effector T-cells

Effector T-cells (T_{EC}) are relatively short-lived activated T-cells and they express neither CD45RO nor CD62L. T_{EC} display an immediate response to infection. In the present study, the percentage of effector T- cells in the PBMCs from patients with ENL and LL controls were investigated to compare the T-cell activation status in patients with ENL reaction and non-reactional LL patient controls before and after treatment.

Nearly one-third (29.3%) of $CD3^+$ T-cells were effector cells in the PBMCs of patients with ENL with the corresponding value of 20.0% in LL patient controls before treatment and the difference was statistically significant ($P \leq 0.001$; $\Delta HL=9.0\%$). After treatment, the median percentage of $CD3^+$ T_{EC} was significantly decreased in patients with ENL (24.6%) compared to in LL patient controls (35.3%) ($P \leq 0.05$); $\Delta HL=8.6\%$). With regard to the median percentage of $CD4^+$ $T_{EC,}$ a statistically significant difference was not obtained between the two groups before, during or after treatment (Figure 6.3.6). It worth mentioning that effector memory T-cells are short-lived unlike memory T-cells and they shortly undergo apoptosis once they meet their cognate antigens.

The median percentage of T_{EC} expression in $CD8^+$ T- cells in patients with ENL was 62.7% which was considerably higher than the value obtained for patients with LL controls (39.5%) before treatment ($P< 0.0001$; $\Delta HL= 25.8\%$). Similar to $CD3^+$ $T_{EC,}$ the proportion of $CD8^+$ T_{EC} in patients with ENL was significantly decreased (38.9%) compared to in LL patient controls (55.2%) after treatment ($P \leq 0.005$; $\Delta HL=14.4$) (Figure 6.3.6).

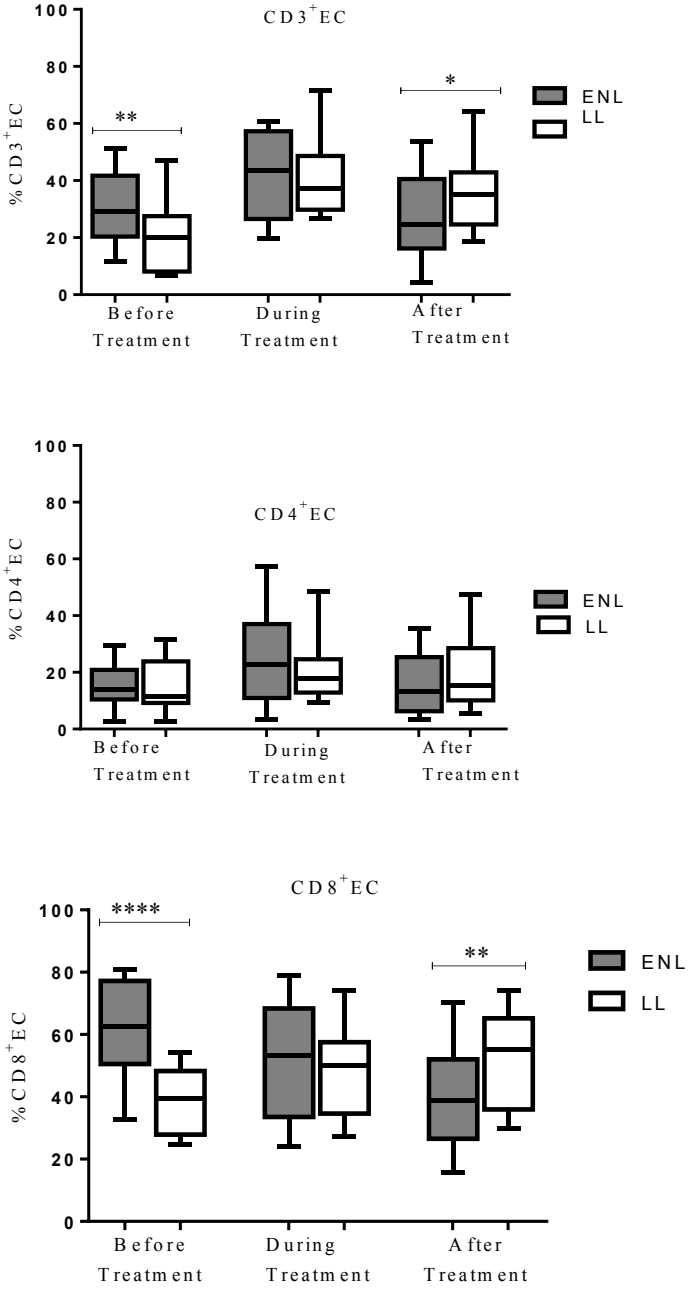


Figure 6.3.6. The median percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells expressing $CD45RO^-CD62L^-$ (T_{EC}) in PBMCs of patients with ENL and LL controls before, during and after prednisolone treatment. n (before) = (during) = n (after) = 35; LL: n (before) = n (during) = (after) =25. Statistical test: Mann-Whitney unpaired test (U). * *P≤0.05*; ** *P≤0.005*; **** *P< 0.0001*. Box and whiskers show median ± interquartile range.

3.2.6. Naive T-cells

A naïve T-cell is a matured differentiated T-cell that has not encountered its cognate antigen within the periphery. Naïve T-cells are usually characterized by the surface expression of L-selectin (CD62L). They do not express memory markers (CD45RO) or activation markers such as CD25, CD44 and CD69. Naïve T-cells do not proliferate until they encounter their corresponding antigens. The relative percentage of naïve T-cells in patients with ENL and LL controls before and after treatment were compared in this study.

Despite the higher bacterial load in patients with LL patients, the median percentage $CD3^+$ naïve T-cells was significantly higher (59.5%) in these patients compared to in patients with ENL (27.7%) before treatment ($P<0.0001$; $\Delta HL= 26.5\%$). During treatment, the median percentage of $CD3^+$ naïve T-cells significantly decreased to 32.9% in LL patient controls while in patients with ENL the proportion was slightly increased to 31.8%. After treatment, the proportion of $CD3^+$ naïve T- cells was further increased to 42.9% in patients with ENL but did not change in LL patient controls (33.0%) and the difference between the two groups was statistically significant ($P \leq 0.05$) (Figure 6.3.7).

The median percentage of $CD4^+$ naïve T- cells in patients with ENL (34.0%) was significantly lower than in LL patient controls (61.5%) before treatment ($P<0.0001$; $\Delta HL=25.6\%$). During treatment the median percentage of $CD4^+$ naïve T- cells was increased in patients with ENL to 45.2% while it was decreased in LL patient controls to 40.0% but the difference was not statistically significant($P>0.05$). After treatment, the median percentage of these cells did not show a significant change (Figure 6.3.7).

Regarding to $CD8^+$ T- cells, the median percentage naïve T-cells in patients with ENL was more than three times lower (15.4%) than in LL patient controls (50.5%) before treatment ($P< 0.0001$; $\Delta HL=31.6\%$). During treatment, while the median percentage of $CD8^+$ naive T-cells increased to 35.5% in patients with ENL, it was decreased to 38% in LL patient controls. After treatment, the median percentage of $CD8^+$ naïve T-cells in patients with ENL and LL controls was 51.5% and 32.8% respectively and the difference between the two groups was statistically significant ($P\leq 0.05$; $\Delta HL=14.4\%$) (Figure 6.3.7).

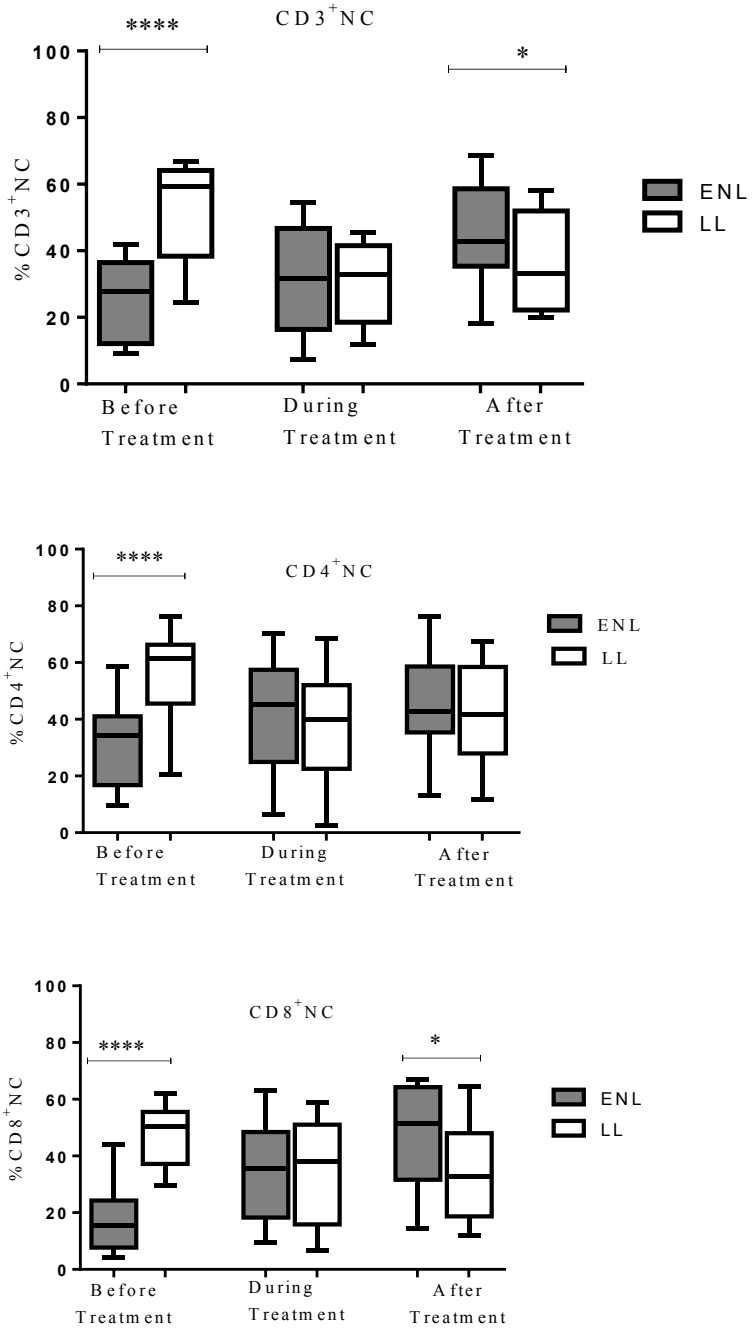


Figure 6.3.7. The median percentage of naïve T-cells expression in $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the PBMCs of patients with ENL and LL controls before, during and after treatment. n (before) = (during) = n (after) = 35; LL: n (before) = n (during) = (after) =25. Statistical test: Mann-Whitney unpaired test (U). * $P \leq 0.05$; **** $P < 0.000$. Box and whiskers show median ± interquartile range.

3.2.7. Treg/T_{EM} cells ratio (ENL versus LL)

The median percentage ratio of regulatory T-cells to effector memory T-cells (Treg/T_{EM} cells) were significantly lower in untreated patients with ENL (0.077) than in LL controls (0.44) at recruitment (P≤ 0.0001). However, after treatment the median percentage ratio of Treg/T_{EM} cells was significantly increased in patients with ENL (0.522) compared LL controls (0.255) (P≤0.005) (Figure 6.3.8).

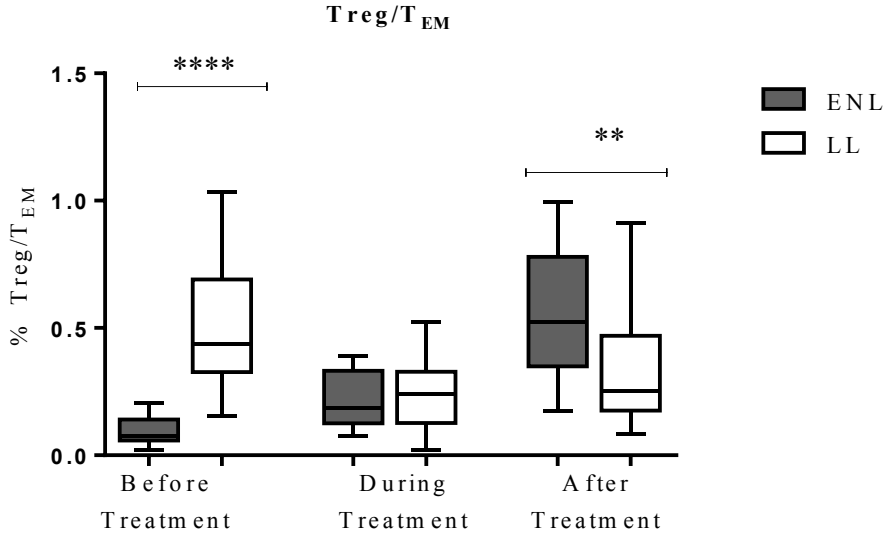


Figure 6.3.8. The median percentage ratio of Treg/T_{EM} cells in the PBMCs of patients with ENL and LL controls before, during and after prednisolone treatment. n (before) = (during) = n (after) = 35; LL: n (before) = n (during) = (after) =25. Statistical test: Mann-Whitney unpaired test (U). ** *P ≤ 0.005*; **** *P< 0.0001*. Box and whiskers show median ± interquartile range.

Table 6.3.1. Summary table sowing trends of memory T-cells before and after treatment of patients with ENL compared to LL controls

Memory T-cells (%)	Before treatment		During treatment		After treatment	
	ENL	LL	ENL	LL	ENL	LL
$CD3^+CD45RO^+$	↑	↓	–	–	–	–
$CD4^+CD45RO^+$	↑	↓	–	–	–	–
$CD8^+CD45RO^+$	–	–	–	–	–	–
$CD3^+CD62L^-$	↑	↓	–	–	–	–
$CD4^+$ $CD62L^-$	↑	↓	–	–	–	–
$CD8^+CD62L^-$	↑	↓	–	–	↓	↑
$CD3^+T_{EM}$	↑	↓	–	–	↓	↑
$CD4^+T_{EM}$	↑	↓	↓	↑	–	–
$CD8^+T_{EM}$	↑	↓	–	–	–	–
$CD3^+T_{CM}$	–	–	–	–		
$CD4^+T_{CM}$	↑	↓	–	–	–	–
$CD8^+T_{CM}$	↑	↓	–	–	–	–
$CD3^+T_{EC}$	↑	↓	–	–	↓	↑
$CD4^+T_{EC}$	–	–	–	–	–	–
$CD8^+T_{EC}$	↑	↓	–	–	↓	↑
$CD3^+T_{NC}$	↓	↑	–	–	↑	↓
$CD4^+T_{NC}$	↓	↑	–	–	–	–
$CD8^+T_{NC}$	↓	↑	–	–	↑	↓

T_{EM} = Effector T-cells, T_{CM}= Central memory T-cells, T_{EC}= effector T-cells, T_{NC}= naïve T-cells. ↑= increased, ↓= decreased, – = no change

3.3. Activated and memory T-cells expression in patients with ENL before and after treatment

The status of activated and memory T- cells in PBMCs of patients with ENL cases before, during and after prednisolone treatment was investigated. Blood samples were obtained from each patient with ENL reactions before, during and after treatment. PBMCs samples were stained with appropriate antibody conjugates as described earlier and the immunophenotyping of activated and memory T-cells was performed by flow cytometry to investigate the pattern of these immune cells during the follow-up period.

3.3.1. Total memory T-cells

The median percentage of $CD3^+$ total memory T-cells ($CD45RO^+$) in patients with ENL was significantly higher (41.1%) before treatment than during treatment (29.2%) ($P \leq 0.001$; $\Delta HL=11.2\%$) and after treatment (31.5%) ($P \leq 0.001$; $\Delta HL=9.8\%$). Similarly, the median percentage of $CD4^+$ total memory T- cells was 52.3% before treatment and significantly decreased to 29.2% during treatment ($P<0.0001$; $\Delta HL=22.1\%$). After treatment, the median percentage of $CD4^+$ memory T- cells increased to 45.0% but still lower than before treatment ($P \leq 0.05$; $\Delta HL=8.75\%$). On the other hand, unlike $CD3^+$ and $CD4^+$ memory T-cells, the median percentage of $CD8^+$ memory T- cells did not change before, during and after treatment (Figure 3.9).

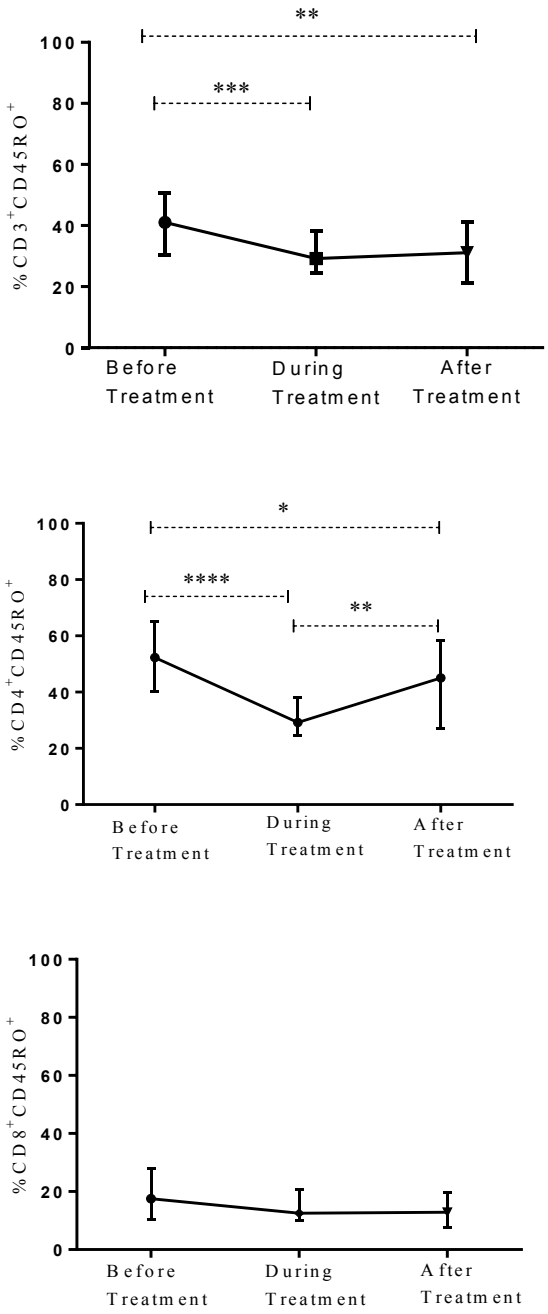


Figure 6.3.9. The median percentage of total memory T-cells ($CD45RO^+$) expression in $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in PBMCs of patients with ENL before, during and after treatment. n (before) = (during) = n (after) = 35; LL: n (before) = n (during) = (after) =25. Statistical test: Wilcoxon matched-pairs test. * $P \leq 0.05$; ** $P < 0.005$; *** $P < 0.001$; **** $P < 0.0001$. . **Error bars** show median ± interquartile range.

3.3.2. Total activated T-cells

The median percentage of $CD3^+$ activated T-cells ($CD62L^-$) before starting prednisolone treatment was 59.2% and it was higher than the percentage obtained during treatment (47.0%) ($P \leq 0.05$). After treatment, the median percentage of activated $CD3^+$ T-cells was decreased to 33.9% which was significantly lower compared with before treatment ($P< 0.0001$; $\Delta HL=25.5\%$). The median percentage of $CD4^+$ activated T-cells before treatment was 50.7% and was decreased to 29.7% during treatment which was statistically significantly different ($P< 0.0001$; $\Delta HL=17.75\%$). After treatment, the proportion of $CD4^+$ activated T-cells remained at 29.0% (Figure 6.3.10).

More than two- third of $CD8^+$ T-cells (71.2%) were $CD62^-$ before treatment. During treatment, the proportion of activated $CD8^+$ T-cells decreased to 59.5% and was lower than before treatment ($P \leq 0.05$). Unlike activated $CD4^+$ T-cells, the median percentage of activated $CD8^+$ T-cells after treatment was significantly lower (34.5%) than before treatment ($P< 0.0001$; $\Delta HL=34.2\%$) (Figure 6.3.10).

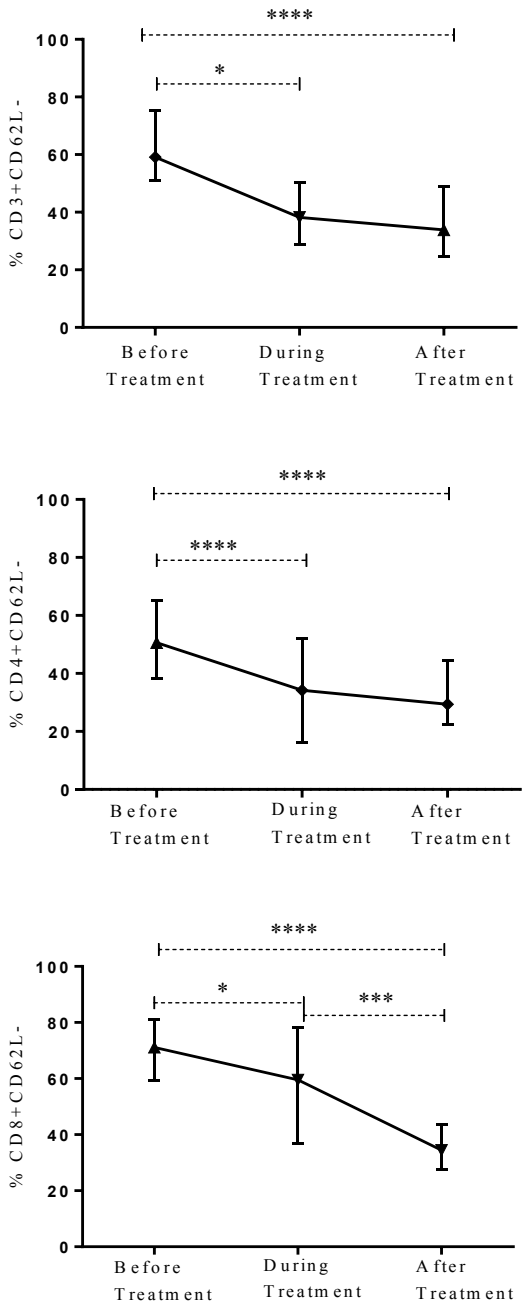


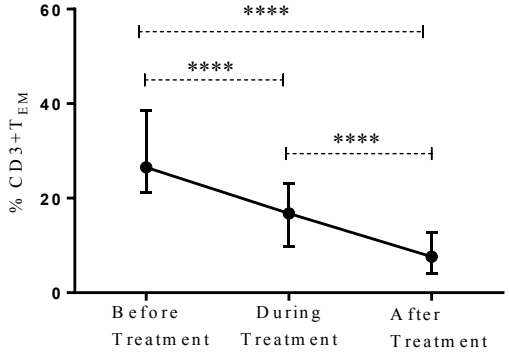
Figure 6.3.10. The median percentage of activated T-cells in $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in PBMCs of patients with ENL before, during and after treatment. n (before) = (during) = n (after) = 35; LL: n (before) = n (during) = (after) =25. Statistical test: Wilcoxon matched-pairs test. * P≤*0.05*; *** *P≤ 0.001*; **** *P < 0.0001.* **Error bars** show median ± interquartile range.

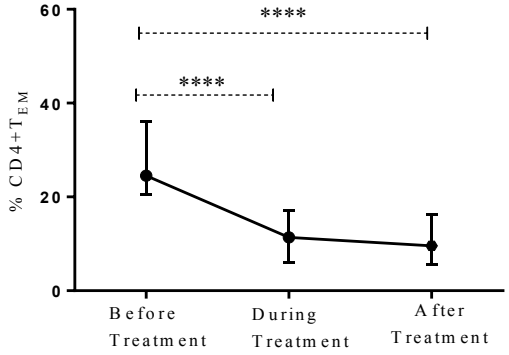
3.3.3. Effector memory T-cells expression

The median percentage of effector (T_{EM}) and central memory T-cells (T_{CM}) before and after treatment of patients ENL were investigated based on the expression of CD62L (a homing receptor) as previously described. T_{CM} cells express CD62L to localize in secondary lymphoid organs. In these secondary lymphoid organs, they are ready to proliferate upon re-encountering antigen. T_{EM} cells do not express CD62L as they circulate in the periphery and have immediate effector functions upon encountering the antigen.

Patients with ENL reactions had higher $CD3^+$ T_{EM} cells (26.6%) before treatment than during treatment (16.8%) ($P< 0.0001$; ΔHL =11.88%). The percentage of $CD3^+$ T-cells expressing T_{EM} cells ($CD3^+CD45RO^+CD62L^-$) was considerably decreased to 7.6% after treatment which was substantially lower than the median percentage of $CD3^+$ T_{EM} cells before treatment ($P<0.0001$; ΔHL=20.0%). Likewise, the median percentage of $CD4^+$ T_{EM} ($CD4^+CD45RO^+CD62L^-$) cells was more than twofold higher (24.6 %) before treatment compared to during treatment (11.4%) and the difference was statistically significant ($P<0.0001$; ΔHL=15.54%). The percentage of $CD4^+$ T_{EM} cells was remarkably reduced to 9.6% after treatment indicating the decreasing tendency of T-cell activation after prednisolone treatment of patients with ENL (Figure 6.3.11).

About 16.5% of $CD8^+$ T-cells ($CD8^+CD45RO^+CD62L^-$) expressed T_{EM} cells before treatment. During treatment, the median percentage of $CD8^+$ T-cells expressing T_{EM} cells was notably decreased to 7.5% which was significantly low compared to before treatment ($P\leq 0.005$; ΔHL=6.61%). However, unlike $CD4^+$ and $CD3^+$ T_{EM} cells, the proportion of $CD8^+$ T_{EM} cells did not show significant change after treatment (6.5%) compared to the figure obtained during treatment (Figure 6.3.11).





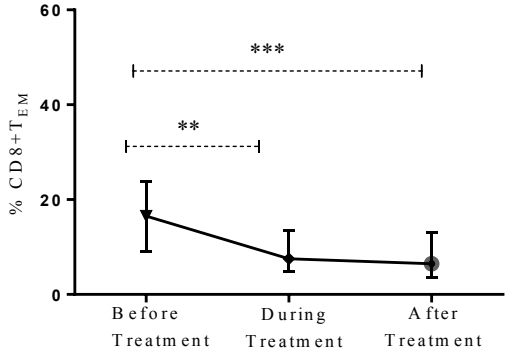


Figure 6.3.11. The median percentage of effector memory T- cells expression in $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in PBMCs of ENL before, during and after treatment. n (before) = (during) = n (after) = 35; LL: n (before) = n (during) = (after) =25. Statistical test: Wilcoxon matched-pairs test. ** *P< 0.005*; *** $P \leq 0.001$; **** *P < 0.0001*. **Error bars** show median ± interquartile range.

3.3.4. Central memory T-cells

Patients with ENL had a higher median percentage of $CD3^+$ T_{CM} cells (13.8%) before treatment than during treatment (9.8%) ($P \leq 0.005$; $\Delta HL=4.1\%$). However, the proportion of $CD3^+$ T-cells expressing T_{CM} ($CD45RO^+CD62L^+$) was considerably increased from 9.8% of during treatment to 13.5% after treatment ($P \leq 0.05$; $\Delta HL=$ 4.6%). Interestingly, unlike the proportion of $CD3^+$ effector memory T-cells, the median percentage of $CD3^+$ central memory T-cells before and after treatment was unchanged (Figure 6.3.13).

Similarly, the proportion of $CD4^+$ T_{CM} was higher (23.5%) before treatment than during treatment (13.7%) and the difference was statistically significant ($P \leq 0.001$; $\Delta HL=9.04\%$). After treatment, the percentage of $CD4^+$ T_{CM} was again increased to 22.0% which was not significantly different compared to the percentage value obtained before treatment (Figure 6.3.13).

The proportion of $CD8^+$ T-cells expressing central memory ($CD45RO^+CD62L^+$) showed an opposite trend. Before treatment, about 1.2% of $CD8^+$ T-cells expressed T_{CM} cells. During treatment the proportion of $CD8^+$ T-cells expressing T_{CM} cells was increased to 1.9% which was significantly higher than before treatment ($P \leq 0.05$; $\Delta HL=0.66\%$). The percentage of $CD8^+$ T_{CM} cells further increased to 2.7% after treatment and it was higher than the percentage value obtained before treatment ($P < 0.0001$; $\Delta HL=1.32\%$). The percentage of $CD8^+$ T_{CM} was not significantly different during and after prednisolone treatment (Figure 6.3.13).

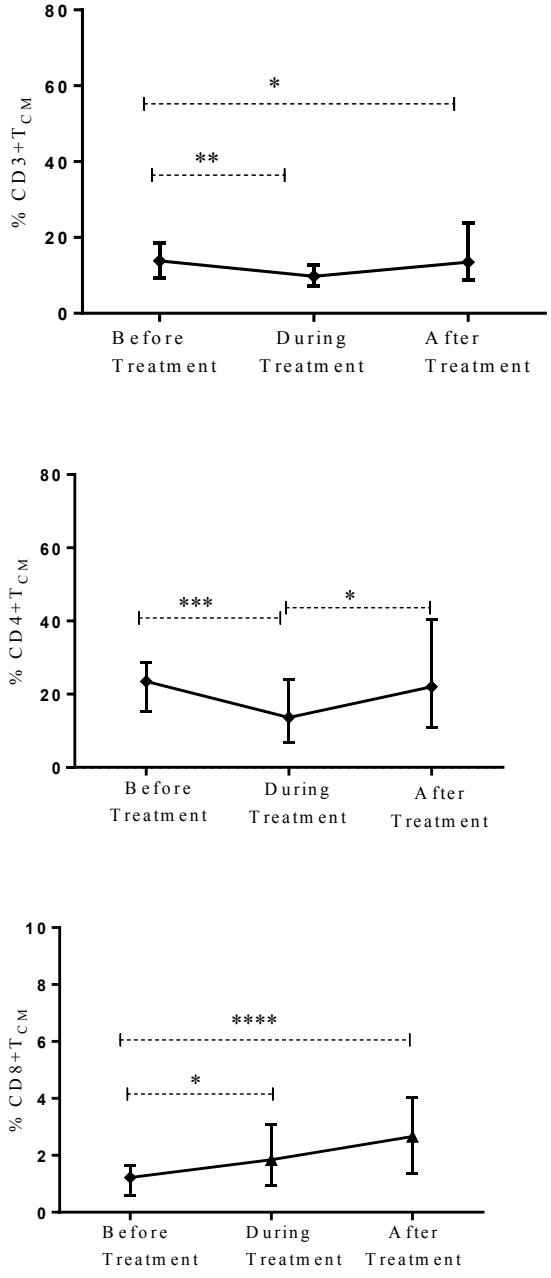


Figure 6.3.12. The median percentage of central memory T cells expression in $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in PBMCs of patients with ENL before, during and after treatment. n (before) = (during) = n (after) = 35; LL: n (before) = n (during) = (after) =25. Statistical test: Wilcoxon matched-pairs test. * *P≤0.05*; ** *P≤ 0.005*; *** *P≤ 0.001*; **** *P< 0.0001*. .**Error bars** show median ± interquartile range.

3.3.5. Effector and naïve T-cells

Effector T-cells (T_{EC}) are terminally differentiated memory T-cells. They are the relatively short-lived activated cells whose functions involve the interaction of an armed effector T-cell with a target cell displaying specific antigen. They neither display memory marker (CD45RO) nor the homing receptor (CD62L). Naïve T-cells display the homing receptor marker but do not display memory marker.

The median percentage of $CD3^+$ T_{EC} cells was lower before treatment (29.3%) than during treatment (43.4%) ($P \leq 0.005$). After treatment, the percentage of these cells decreased to 24.6%. Similarly, the median percentage of $CD4^+$ T_{EC} cells was lower before treatment (14.0%) than during treatment (22.9%) ($P \leq 0.05$). After treatment, the percentage of $CD4^+$ T_{EC} cells was decreased by half (12.1%) than during treatment ($P \leq 0.05$). Like $CD3^+$ T_{EC} cells, the percentage of $CD4^+$ T_{EC} cells did not show significant difference before and after treatment (Figure 6.3.12). On the other hand, the median percentage of $CD8^+$ T_{EC} cells was slightly higher (62.7%) before treatment than during treatment (53.4%) ($P \leq 0.05$). After treatment, the percentage of $CD8^+$ T_{EC} cells was considerably decreased to 38.9% compared to the figure obtained during treatment ($P \leq 0.05$) and before treatment ($P < 0.0001$) (Figure 6.3.13).

With regard to naïve T-cells (T_{NC}), about 27.7% and 31.8% of $CD3^+$T-cells was T_{NC} cells before and during treatment respectively and the difference was not statistically significant. However, after treatment, the median percentage of $CD3^+T_{NC}$ cells noticeably increased to 42.9% compared to during treatment ($P \leq 0.005$) and after treatment ($P < 0.0001$). Similarly, the percentage of $CD4^+$ T_{NC} cells was lower (34.1%) before treatment than during treatment (45.2%) ($P \leq 0.005$). After treatment, the percentage of $CD4^+T_{NC}$ cells (40.4%) did not show a statistically significant difference compared to the value obtained during treatment but was significantly higher than that of before treatment ($P \leq 0.005$). The percentage of $CD8^+$ T_{NC} cells was 15.4% before treatment and it was appreciably increased to 35.5% during treatment ($P \leq 0.001$). The percentage of these $CD8^+T_{NC}$ cells was further increased to 51.5%, which was significantly higher than the value measured (35.5%) during treatment ($P \leq 0.05$). The median percentage of $CD8^+$ T_{NC} cells was significantly higher after treatment than before treatment ($P < 0.0001$) (Figure 6.3.14).

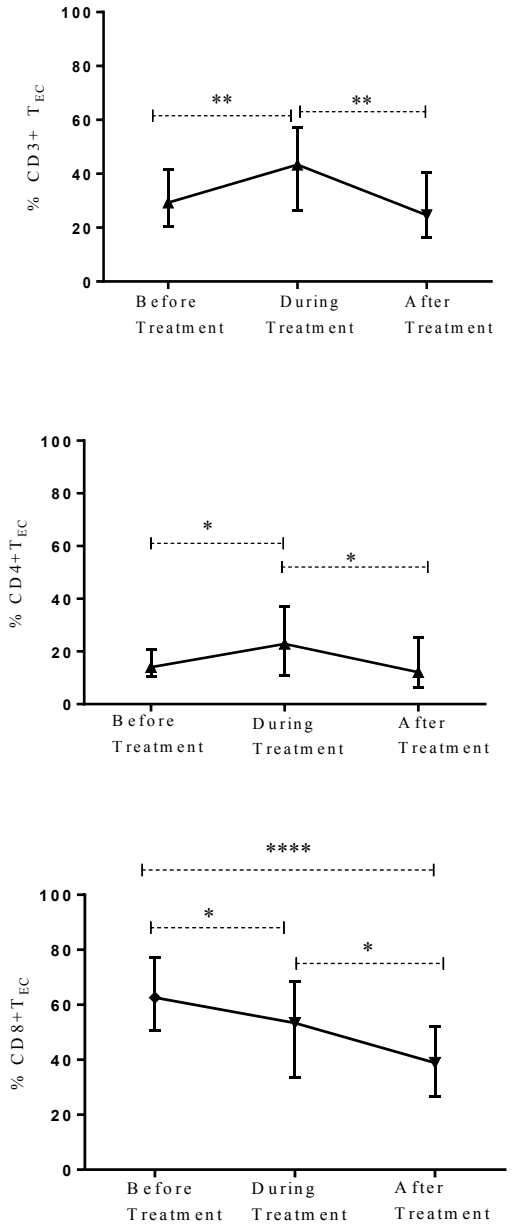


Table 6.3.13. The median percentage of effector T-cells expression in $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in PBMCs of patients with ENL before, during and after treatment. n (before) = (during) = n (after) = 35; LL: n (before) = n (during) = (after) =25. Statistical test: Wilcoxon matched-pairs test. * *P≤0.05*; ** *P≤ 0.005*; *** *P≤ 0.001*; **** *P< 0.0001*. . **Error bars** show median ± interquartile range.

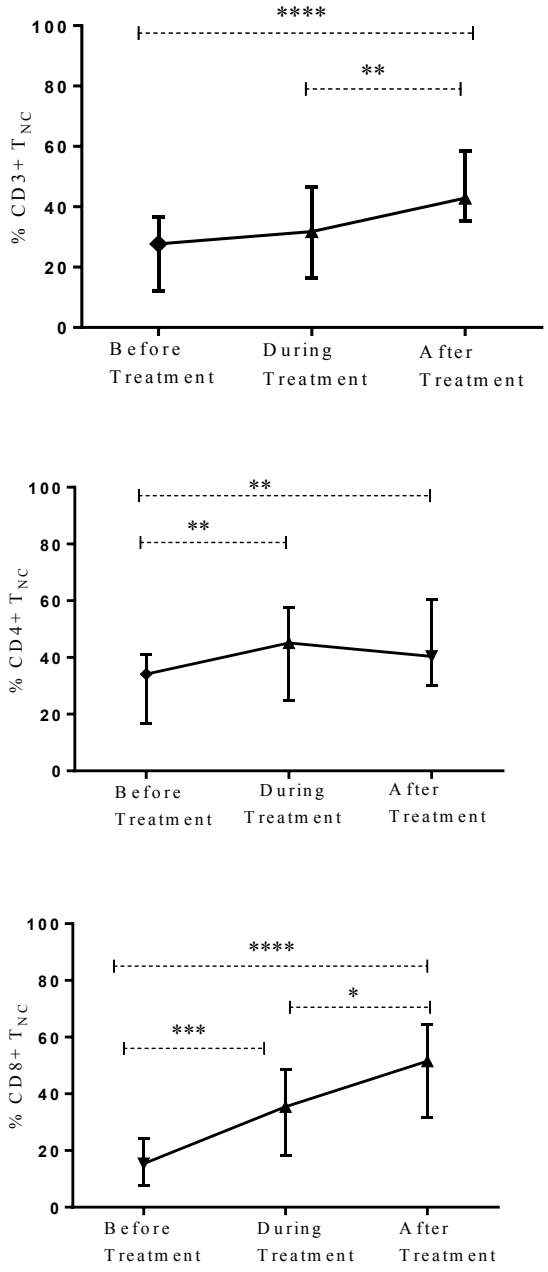


Figure 6.3.14. The median percentage of naïve $CD3^+$, $CD4^+$ and $CD8^+$ T-cells expression in PBMCs of patients with ENL before, during and after treatment. n (before) = (during) = n (after) = 35; LL: n (before) = n (during) = (after) =25. Statistical test: Wilcoxon matched-pairs test. * $P \leq 0.05$; ** $P \leq 0.005$; *** $P \leq 0.001$; **** $P < 0.0001$. . **Error bars** show median ± interquartile range.

3.3.6. Treg/T_{EM} cells ratio in patients with ENL

Patients with ENL had a significantly lower median percentage of Treg/T_{EM} cells (0.077) before treatment than after treatment (0.552) ($P< 0.0001$).

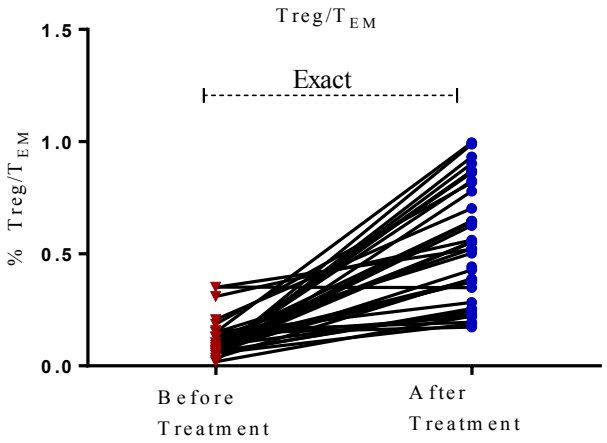


Figure 6.3.15. The median percentage of Treg/T_{EM} cells in the PBMCs of patients with ENL before and after prednisolone treatment. n (before) = (during) = n (after) = 35; LL: n (before) = n (during) = (after) =25. Statistical test: Wilcoxon matched-pairs test. **** *P < 0.0001*.

Table 6.3.2. Summary table sowing trends of memory T-cells before and after treatment of ENL cases

Memory T-cells (%)	Before treatment	After treatment
$CD3^+CD45RO^+$	↑	↓
$CD4^+CD45RO^+$	↑	↓
$CD8^+CD45RO^+$	–	–
$CD3^+CD62L^-$	↑	↓
$CD4^+ CD62L^-$	↑	↓
$CD8^+CD62L^-$	↑	↓
$CD3^+T_{EM}$	↑	↓
$CD4^+T_{EM}$	↑	↓
$CD8^+T_{EM}$	↑	↓
$CD3^+T_{CM}$	–	–
$CD4^+T_{CM}$	–	–
$CD8^+T_{CM}$	↓	↑
$CD3^+T_{EC}$	–	–
$CD4^+T_{EC}$	–	–
$CD8^+T_{EC}$	↑	↓
$CD3^+T_{NC}$	↓	↑
$CD4^+T_{NC}$	↓	↑
$CD8^+T_{NC}$	↓	↑

T_{EM} = Effector T-cells, TCM= Central memory T-cells, TEC= effector T-cells, TNC= naïve T-cells. ↑= increased, ↓= decreased, – = no change

SECTION 4. REGULATORY AND MEMORY T-CELLS IN ACUTE AND CHRONIC ENL

The subgroup analysis between acute and chronic ENL was done to see any significant differences with respect to regulatory and memory T-cells. Although chronic ENL in this study was defined as an ENL occurring for 24 weeks or more during which a patient has required ENL treatment either continuously or where any treatment free period has been 27 days or less, we included only those patients who were off steroid treatment for 15 days or more to exclude the confounding effect of steroid on the outcome variables as defined previously in the material and methods section.

4.1. $CD4^+$ and $CD8^+$ T-cells

The median percentage of $CD4^+$ T cells in acute ENL was slightly higher (64.6%) than in chronic ENL (60.1%) ($P \leq 0.05$). In contrast to $CD4^+$ T-cells the median percentage of $CD8^+$ T-cells in acute ENL was lower (24.2%) than in chronic ENL (32.3%) ($P \leq 0.005$). Acute ENL cases had a significantly higher $CD4^+/CD8^+$ ratio (2.6) than chronic cases (1.9) ($P=0.03$) (Figure 6.4.1).

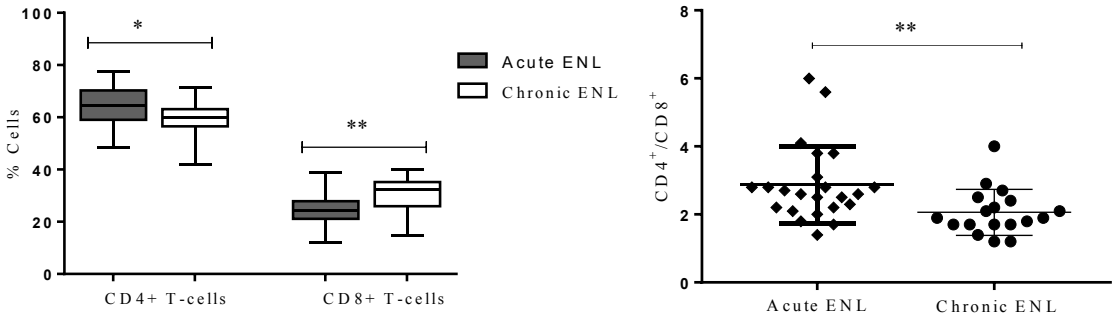


Figure 6.4.1. Median percentage of $CD4^+$ and $CD8^+$ T-cells in acute and chronic ENL and the $CD4^+/CD8^+$ T cell ratio in acute and chronic ENL. n (acute) =23, n (chronic)=18. Statistical test: non parametric, Mann -Whitney (U) test, $\alpha = 0.05$. *$P \leq 0.05$, **$P \leq 0.005$. Box and error bars show median ± interquartile range.

4.2. CD25 and FoxP3 expression in $CD4^+$ and $CD8^+$ T cells

The median percentage of $CD4^+$ $CD25^+$ T-cells in acute and chronic ENL was 8.9% and 8.6% respectively and the difference between the two groups was not statistically significant ($P \geq 0.05$). Similarly, the median percentage of $CD8^+CD25^+$ T-cells in acute ENL was 2.6% while it was 2.6% in chronic ENL ($P \geq 0.05$). The median percentage of $CD4^+$ $FoxP3^+$ T cells in acute ENL (2.3%) and in chronic ENL (2.4%) was not statically significant in the two groups (Figure 6.4.2).

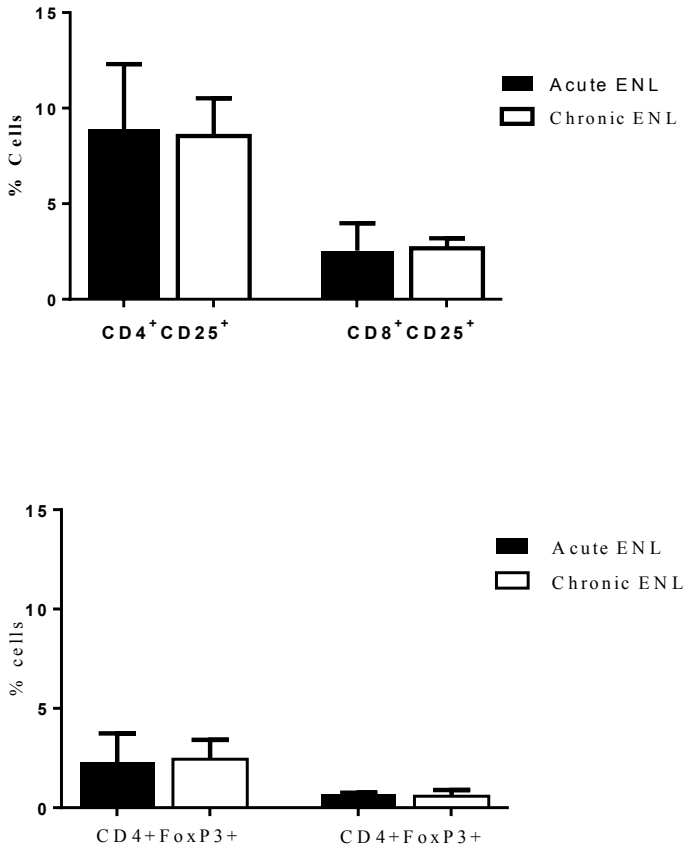


Figure 6.4.2. Median percentage of CD25 and FoxP3 expression in $CD4^+$ and $CD8^+$ T-cells in acute and chronic ENL. n (acute) =23, n (chronic)=18. Statistical test: non parametric, Mann -Whitney (U) test, $\alpha=0.05$. Bar graphs show median ± interquartile range.

4.3. CD25FoxP3 double positives in $CD4^+$ and $CD8^+$ T-cells

About 2.1 % of $CD4^+$ T-cells were expressed $CD25^+FoxP3^+$ in acute ENL cases and 1.8% $CD4^+$ T-cells were express $CD25^+FoxP3^+$ in chronic ENL and the difference in the two groups was not statistically significant ($P \geq 0.05$). Similarly, the median percentage of CD25FoxP3 double positives expression in $CD4^+$ T-cells was 0.69% and 0.27% in acute and chronic ENL respectively ($P \geq 0.05$) (6.4.3).

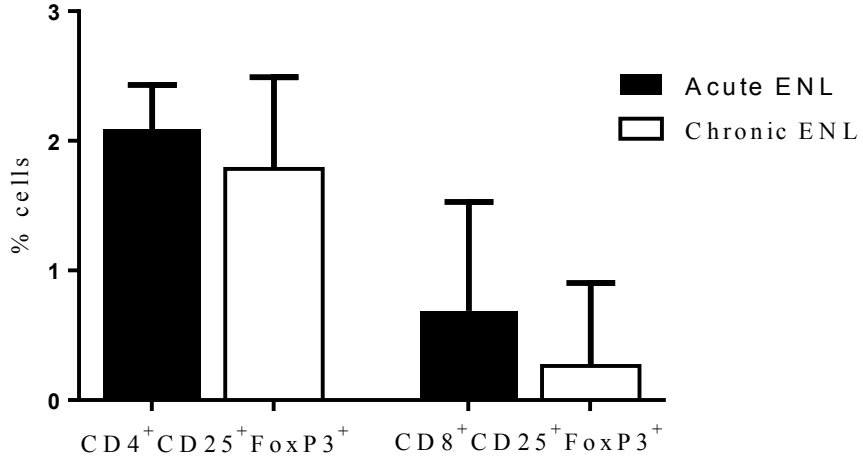


Figure 6.4.3. Median percentage of CD25 FoxP3 positives in CD4+ and CD8+ T-cells in acute and chronic ENL. n (acute) =23, n (chronic)=18. Statistical test: non parametric, Mann -Whitney (U) test, $\alpha=0.05$. Bar graphs show median ± interquartile range.

4.4. $CD4^+$ and $CD8^+$ Tregs

The median percentage expression of $CD4^+$ regulatory T- cells was 1.8% in acute ENL and 1.6% in chronic ENL. The median percentage of $CD8^+$ Tregs was slightly less in Chronic ENL (0.25%) than in acute (0.60) but was not significantly different ($P \geq 0.05$) (Figure 6.4.4).

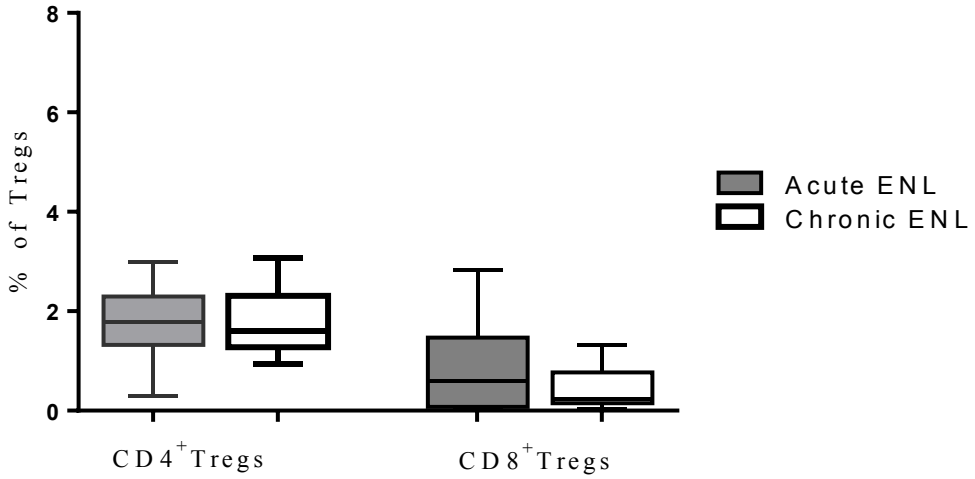


Figure 6.4.4. Median percentage of $CD4^+$ and $CD8^+$ Tregs in acute and chronic ENL. n (acute) =23, n (chronic) =18. Statistical test: non parametric, Mann - Whitney (U) test, α=0.05. Box and whiskers show median ± interquartile range.

4.5. Effector and central memory T-cells

The median percentage of $CD3^+$ effector memory T-cells (T_{EM}) in acute ENL was 31.4% while it was 25.2% in Chronic ENL. Acute ENL cases had 24.0% $CD4^+T_{EM}$ while chronic ENL cases had 31.9% $CD4^+T_{EM}$. The median percentage of $CD8^+$ T_{EM} in acute and chronic ENL was 17.8% and 15.7% respectively. The median percentage of T_{EM} was not significantly different in acute and chronic ENL in all investigated T-cell subsets (Figure 6.4.5). Similarly, acute and chronic ENL had similar median percentage of central memory T-cell (T_{EM}) expression in $CD3^+$, $CD4^+$, and $CD4^+$ T-cells (Figure 6.4.5).

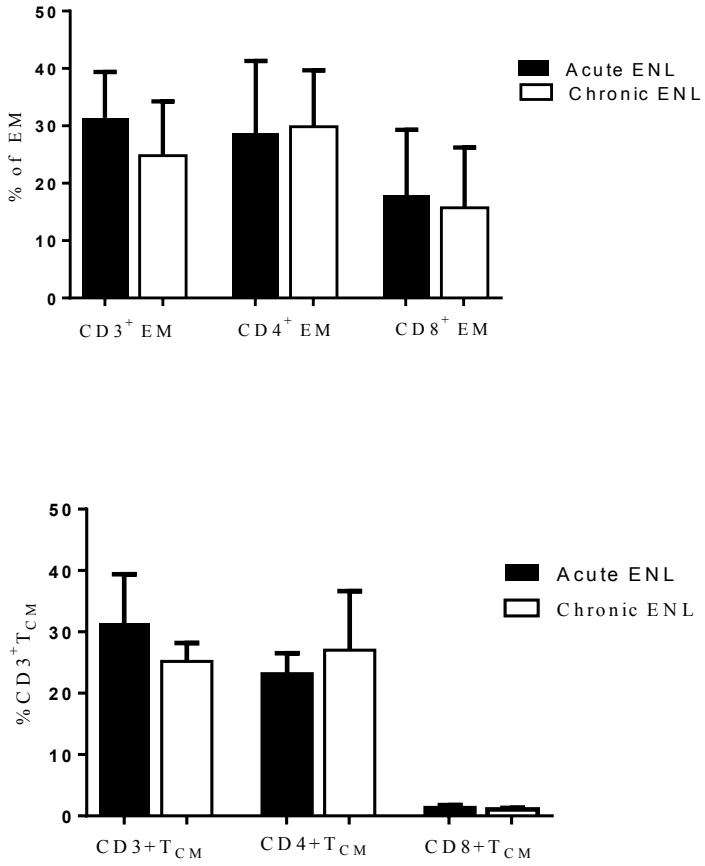


Figure 6.4.5. Median percentage of T_{EM} and T_{CM} expression in $CD3^+$,$CD4^+$ and $CD8^+$ T-cells in acute and chronic ENL. n (acute) =23, n (chronic) =18. Statistical test: non parametric, Mann -Whitney (U) test, α=0.05. Bar graphs show median ± interquartile range.

4.6. Effector and naive T-cells

The median percentage of $CD3^+$ effector T-cells (T_{EC}) in acute ENL was significantly lower (26.6%) than in chronic ENL (41.1%) ($P \leq 0.00$). Similarly, acute ENL cases had a significantly lower median percentage of $CD8^+T_{EC}$ (58.7%) than chronic ENL (73.9%) ($P \leq 0.005$). However, the median percentage of $CD4^+T_{EC}$ (13.6%) in acute and chronic (17.6%) ENL was not significantly different (Figure 6.4.6). The median percentage of naïve T- cell expression in CD3+, $CD4^+$ and $CD8^+$ T-cells in acute and chronic ENL was not significantly different (Figure 6.4.6).

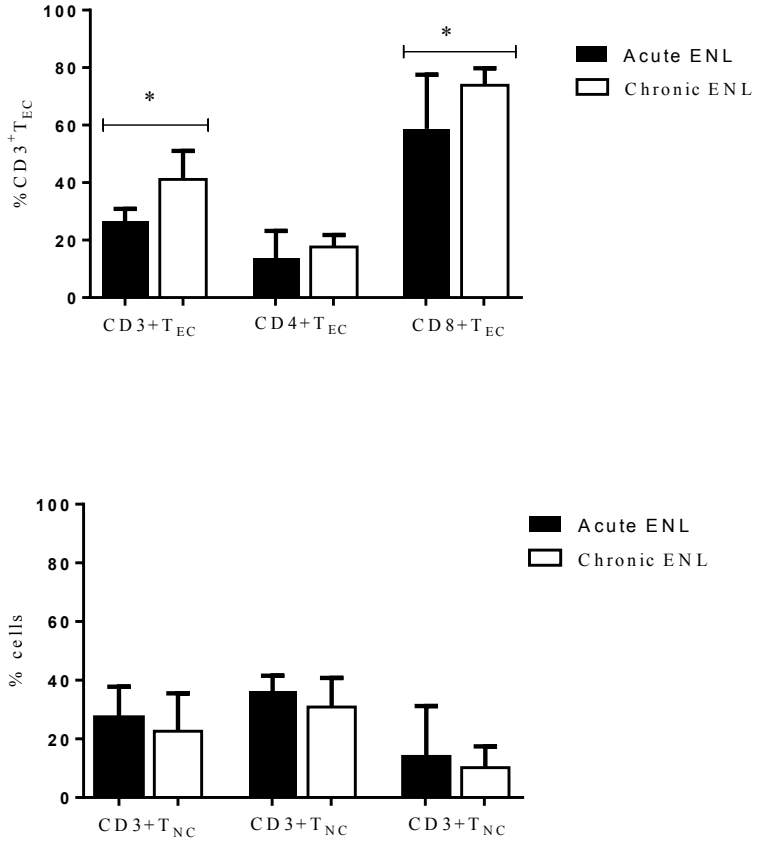


Figure 6.4.6. Median percentage of T_{EC} and T_{NC} expression in $CD3^+$,$CD4^+$ and $CD8^+$ T-cells in acute and chronic ENL. n (acute) =23, n (chronic) =18. Statistical test: non parametric, Mann -Whitney (U) test, $\alpha=0.05$. * $p \leq 0.05$. Bar graphs show median ± interquartile range.

SECTION 5: MEMORY B-CELLS

5.1. Introduction

Memory B-lymphocytes are a sub-type of B-cells that are formed within germinal centres following infection. They proliferate and differentiate into antibody secreting plasma cells also called effector B-cells in response to reinfection. Recent advances in tracking antigen-experienced memory B- cells have revealed the importance of distinct classes of memory B-cells in the development of vaccines and therapeutics. Although in several studies the antibodies production in leprosy have been described, B-cells are among the least studied immune cells in leprosy and information is lacking on the role of B-cells in the pathogenesis of leprosy particularly leprosy reactions. This study provided basic preliminary information with regard to the different classes of B-cells for the first time in patients with ENL and LL controls.

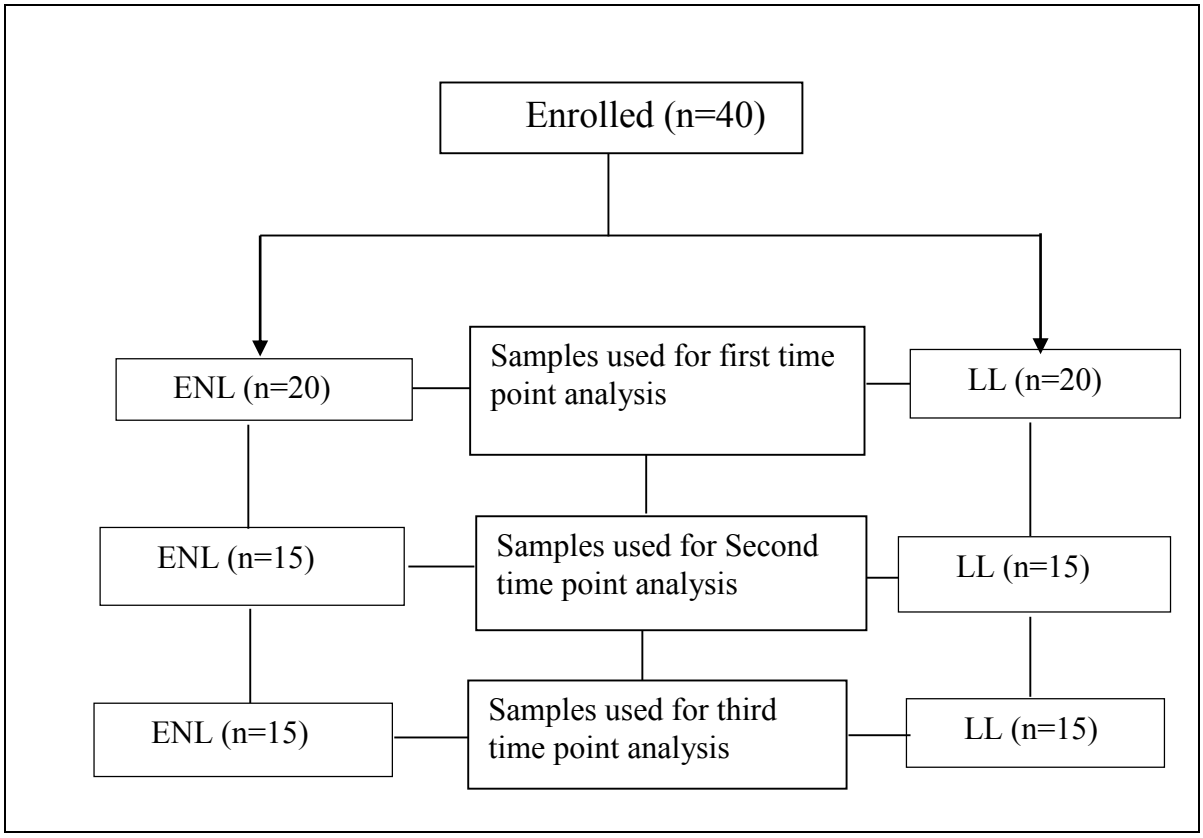


Figure 6.5.1. Flowchart showing the number of samples used for phenotyping of B-cells by flow cytometry.

5.2. Total B- lymphocytes and mature B-cells in patients with ENL and LL controls

The proportion of B-lymphocytes in patients with ENL (9.5%) and LL controls (11.6%) was not statistically significantly different at recruitment. During treatment, the percentage of B-cells slightly increased to 10% in patients in ENL cases and to 14% in LL patient controls but did not show a statistically significant difference. However, after treatment, the proportion of B-cells appreciably decreased to 5.7% in patients with ENL while it was remained at the same level in LL patient controls (12.0%) and the difference between the two groups was statistically significantly different ($P \leq 0.001$; $\Delta HL = 6.02\%$) (Figure 6.5.2).

The median percentage of mature ($CD19^{+}CD10^{-}$) and immature ($CD19^{+}CD10^{+}$) B-cells in patients with ENL and LL controls before and after treatment were also measured. A significant difference was not observed with regard to the proportion of mature and immature B-cells in patients with ENL cases and LL controls before and after treatment (Figure 6.5.2).

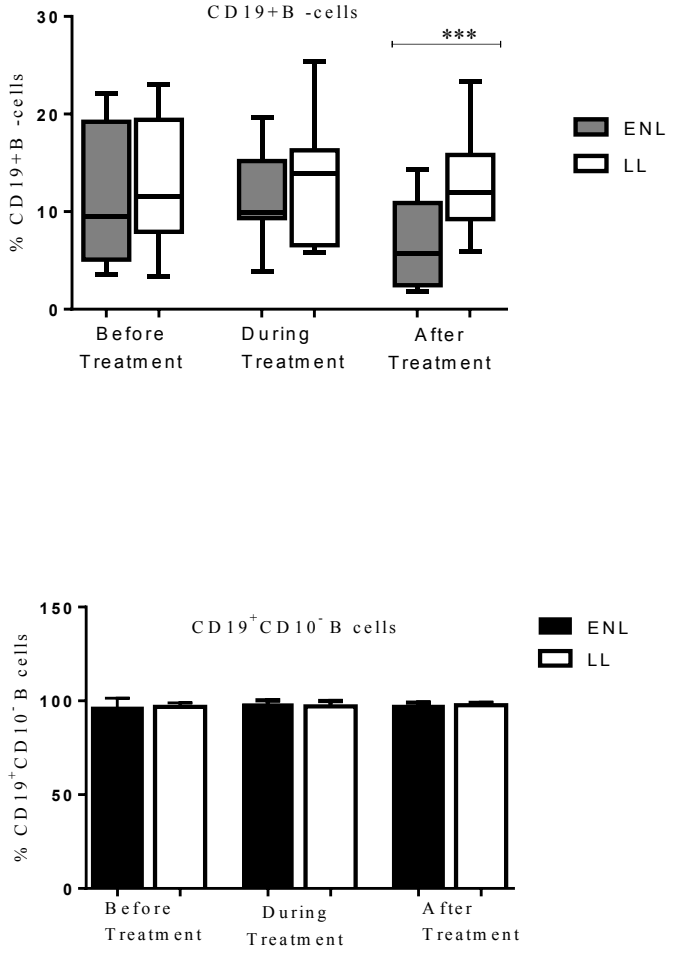


Figure 6.5.2. Total B- lymphocytes ($CD19^{+}$) and mature B -cells ($CD19^{+}CD10^{-}$) production in the PBMCs of patients with ENL and LL controls before, during and after treatment. ENL: n (before) =20, n (during) = n (after) = 15; LL: n (before) = 20, n (during) = (after) =15. Statistical test: Mann-Whitney unpaired test (U). *** $P \leq 0.001$. Box and whiskers show median ± interquartile range.

5.3. Memory B-cells in patients with ENL and LL controls

The median percentage expression of memory B-cell subtypes (resting, activated and tissue-like memory B-cells) and naïve B-cells in patients with ENL and LL controls before and after treatment was analysed. The median percentage of naïve B-cells (NB) was lower in patients with ENL (76.0%) than in LL patient controls (84.6%) before treatment ($P \leq 0.05$; $\Delta HL=6.75\%$). The proportion of these cells decreased to nearly 64% in both cases during treatment and slightly increased to 74.3% in patients with ENL and to 71.6% in LL patient controls after treatment but was not statistically significantly different (Figure 6.5.3).

The median percentage of resting memory B-cells ($CD19^{+}CD27^{+}CD21^{+}$) was 5.8 % in patients with ENL and 4.8 % in LL patient controls before treatment and it was not significantly different in both groups. However, during treatment it was increased to 15.2 % in patients with ENL and was higher than in LL patient controls (8.6%) ($P \leq 0.05$; $\Delta HL=6.95\%$). The proportion of these memory cells was decreased to below 5% in both groups after treatment (Figure 6.5.3).

The median percentage of activated memory B-cells (AM) was slightly higher in patients with ENL (2.1%) than in LL patient controls (1.4%) before treatment but the difference was not statistically significant. During treatment, the percentage AM cells in patients with ENL and LL controls increased to 3.8% and 4.4% respectively and the difference was not statistically significant ($P > 0.05$). After treatment, the percentage of these memory cells did not change (Figure 6.5.4).

The percentage of tissue-like memory B-cells (TLM) was significantly higher in patients with ENL (9.3%) that in LL patient controls (4.8%) before treatment ($P \leq 0.05$). However, during and after treatment, the percentage of TLM in patients with ENL and LL controls was not significantly different (Figure 6.5.4).

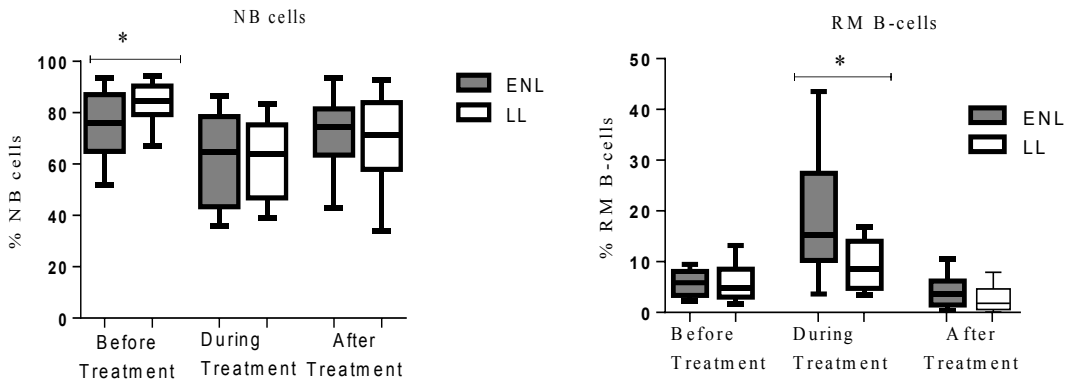


Figure 6.5.3. The median percentage of naïve (NB) B-lymphocytes ($CD19^{+}CD10^{-}CD27^{-}CD21^{+}$) and resting memory (RM) B-cells ($CD19^{+}CD10^{-}CD27^{+}CD21^{+}$) in the PBMCs of patients with ENL and LL controls before, during and after treatment. ENL: n (before) =20, n (during) = n (after) = 15; LL: n (before) = 20, n (during) = (after) =15. Statistical test: Mann-Whitney unpaired test (U). * $P \leq 0.05$.

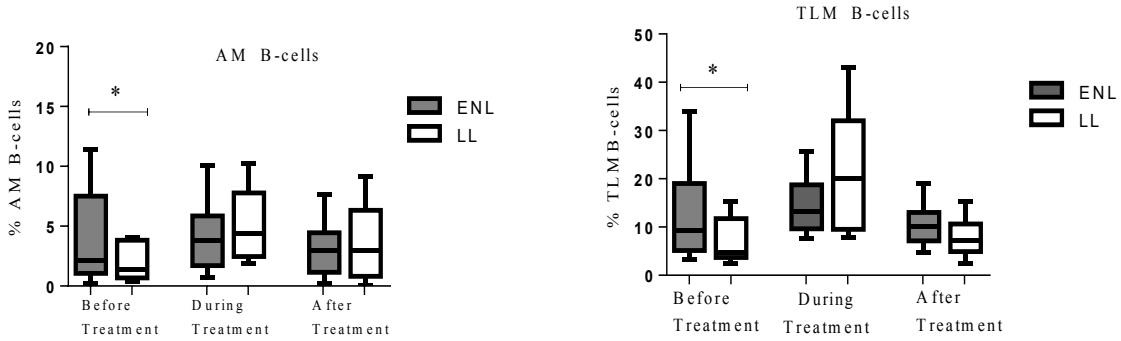


Figure 6.5.4. The median percentage of activated memory (AM) B-cells ($CD19^{+}CD10^{-}CD27^{+}CD21^{-}$) and tissue like memory (TLM) B-cells ($CD19^{+}CD10^{-}CD27^{-}CD21^{-}$) in the PBMCs of patients with ENL and LL controls before, during and after treatment. ENL: n (before) =20, n (during) = n (after) = 15; LL: n (before) = 20, n (during) = (after) =15. Statistical test: Mann-Whitney unpaired test (U). * $P \leq 0.05$. Box and whiskers show median ± interquartile range.

5.4. Total B- lymphocytes and matured B-cells in patients with ENL before and after treatment

The median percentage of B-lymphocytes before and during treatment of patients with ENL was 9.5% and 9.9% respectively. After treatment, the proportion of these cells was significantly decreased to (5.7%) compared with before and during treatment ($P \leq 0.05$). However, the percentage of mature B-cells was slightly lower before treatment than during treatment ($P \leq 0.05$). The proportion of mature B-cells before and after treatment was not statistically significantly different (Figure 6.5.5).

The median percentage of naïve B-cells among untreated patients with ENL (76.0%) was higher than during treatment (64.7%) ($P \leq 0.05$). Interestingly, unlike the central memory T-cells, the proportion of resting memory B-cells (RM) was considerably lower (5.8%) before treatment than during treatment (15.3%) ($P \leq 0.001$). After treatment, the proportion of these resting memory B-cells was decreased to 3.7% and it was significantly lower than before and during treatment ($P \leq 0.05$) (Figure 6.5.6).

The median percentage of activated memory B-cells (AM) was slightly lower (2.1%) before treatment than during treatment (3.8%) but was not significantly different. After treatment, the percentage of AM did not change. Similarly, the percentage of tissue-like memory B-cells (13.2%) was higher before treatment than after treatment (10.0%) but was not statistically significantly different (Figure 6.5.6).

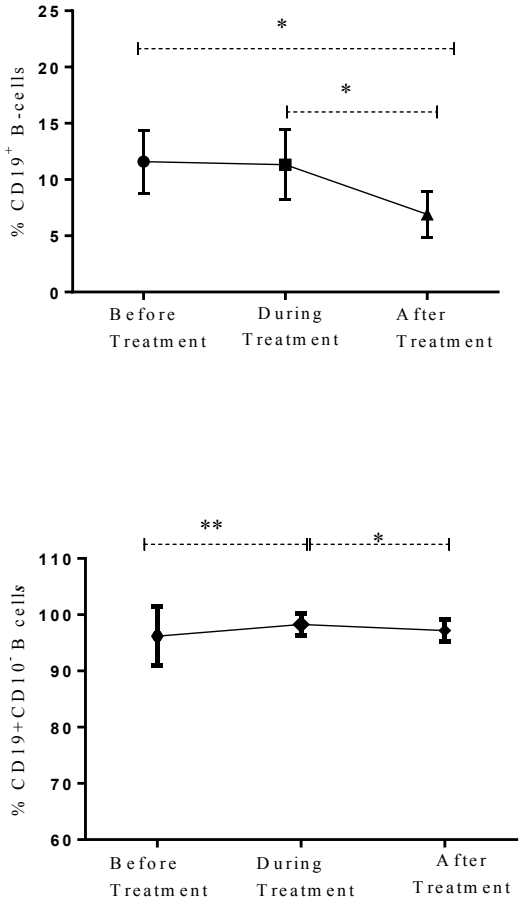


Figure 6.5.5. Total B-lymphocytes ($CD19^+$) and matured B-cells ($CD19^+CD10^-$) expression in PBMCs of patients with ENL before, during and after prednisolone treatment. ENL: n (before) = (during) = n (after) = 15; LL: n (before) = n (during) = (after) =15. Statistical test: Wilcoxon matched-pairs test. * $P \leq 0.05$; ** $P \leq 0.005$. Error bars show median ± interquartile range.

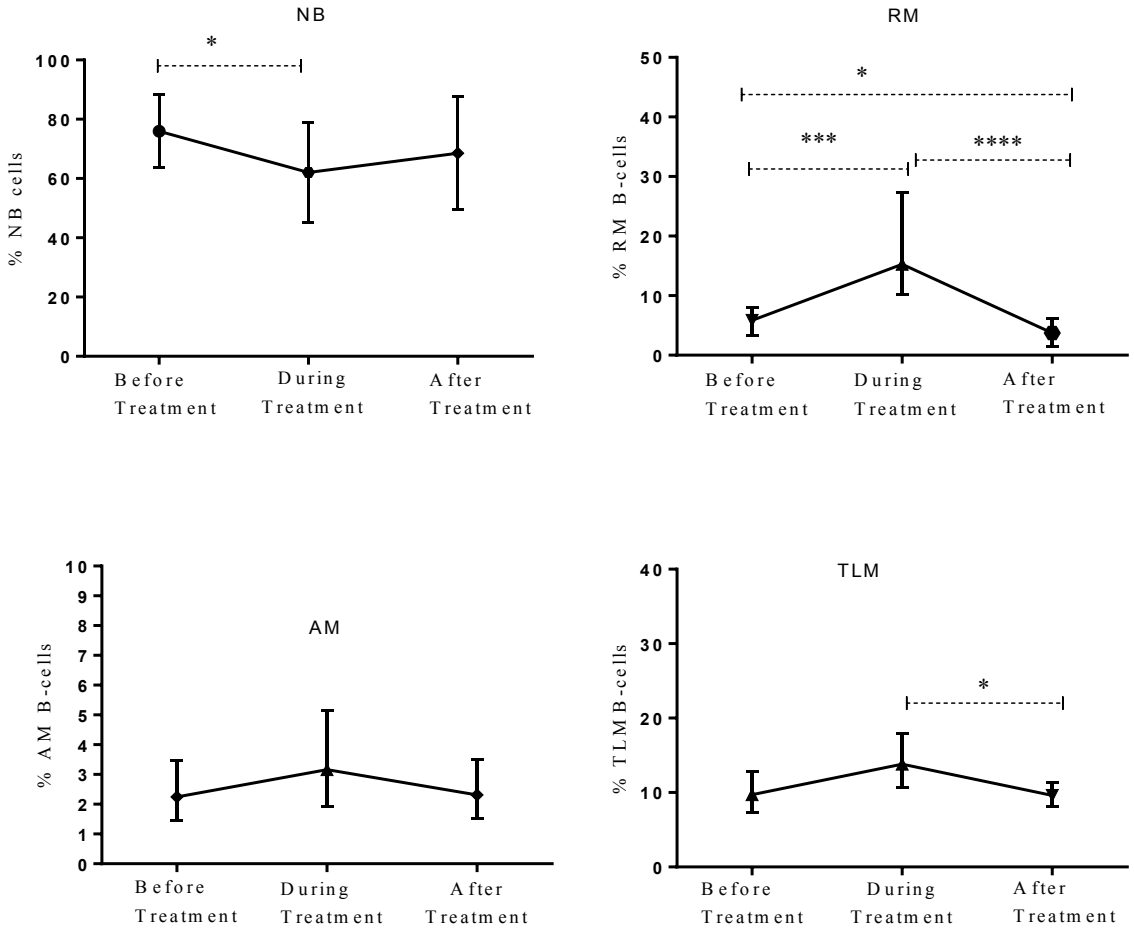


Figure 6.5.6. Memory B-cells: Total naive (NB) B-lymphocytes ($CD19^{+}CD10^{-}CD27^{-}CD21^{+}$), resting memory (RM) B-cells ($CD19^{+}CD10^{-}CD27^{+}CD21^{+}$), expression of activated memory (AM) B-cells ($CD19^{+}CD10^{-}CD27^{+}CD21^{-}$) and tissue like memory (TLM) B -cells ($CD19^{+}CD10^{-}CD27^{-}CD21^{-}$) in the PBMCs of patients with ENL before, during and after prednisolone treatment. ENL: n (before) = (during) = n (after) = 15; LL: n (before) = n (during) = (after) =15. Statistical test: Wilcoxon matched-pairs test. * $P \leq 0.05$; ** $P \leq 0.005$; *** $P \leq 0.001$; **** $P < 0.0001$. Error bars show median ± interquartile range.

Table 6.5.1. Summary tables sowing trends of memory B-cells in patients with ENL and LL controls: A. ENL versus LL, B. within ENL

A.

B-cells	Before treatment		During treatment		After treatment	
	ENL	LL	ENL	LL	ENL	LL
$CD19^+$	–	–	–	–	↓	↑
$CD19^+$ $CD10^-$	–	–	–	–	–	–
AM	↑	↓	–	–	–	–
RM	–	–	↑	↓	–	–
TLM	↑	↓	–	–	–	–
NB	↓	↑	–	–	–	–

B.

B-cells	Before and After treatment	
	Before	After
$CD19^+$	↑	↓
$CD19^+$ $CD10^-$	–	–
AM	–	–
RM	↑	↓
TLM	–	–
NB	–	–

$CD19^+$ marker for B-cells, $CD19^+$ $CD10^-$= marker for mature B-cells, AM= Activated memory B-cells, RM= Resting memory B-cells, TLM= Tissue- like Memory B-cells, NB= naïve B-cells. ↑= increased, ↓= decreased, – = no change

SECTION 6: *IN VITRO* CYTOKINE PRODUCTION

To investigate the *in vitro* cytokine response to *M. leprae*, PBMCs from patients with ENL and LL controls were cultured with *M. leprae* whole cell sonicate (MLWCS), Phytohaemagglutinin (PHA) or left unstimulated for 6 days. Phytohaemagglutinin (PHA) and plain media were used as positive and negative controls respectively. The cytokine levels in culture supernatants were determined using sandwich ELISAs specific to TNF-α, IFN-γ, IL-β, IL-6, IL-8, IL-17A and IL-10. The assay sensitivity for each cytokine is given in materials and methods (Chapter 5). Differences in cytokine production in patients with ENL and LL controls were investigated using an unpaired t-test while the cytokine production among patients with ENL before and after treatment was determined by a paired t-test at 5% level of significance.

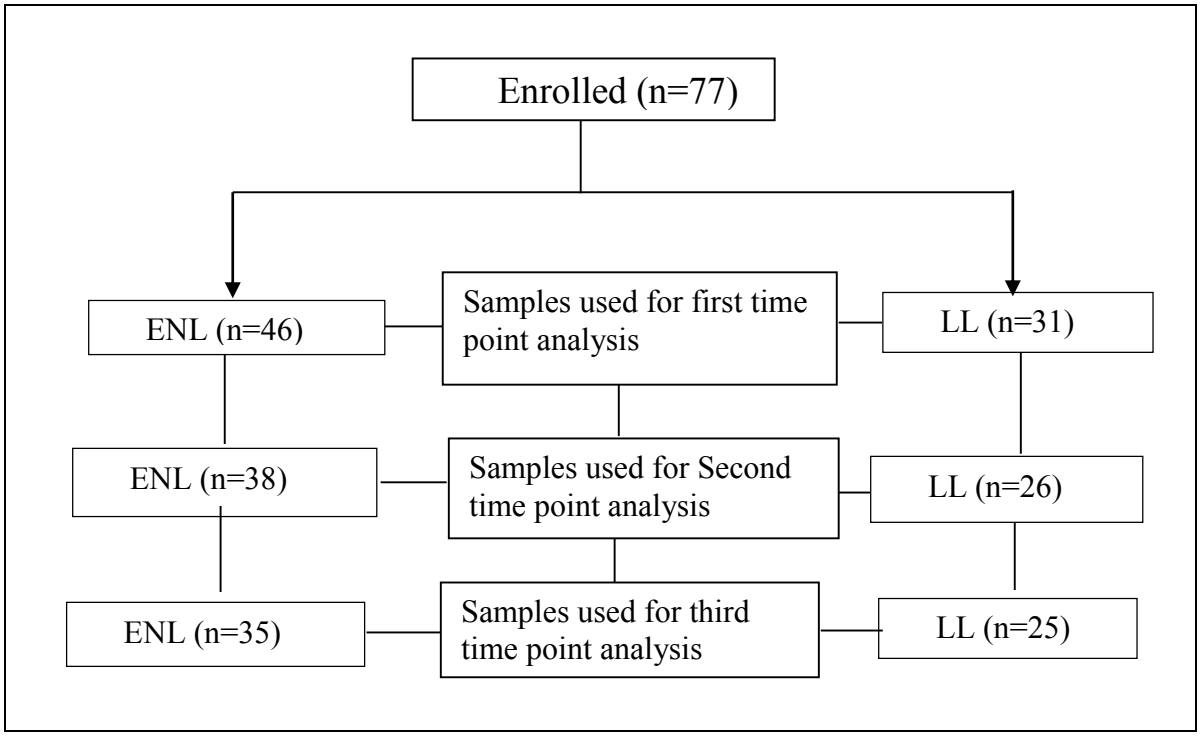


Figure 6.6.1: Flow diagram showing number of samples used for *in vitro* cytokine response to *M. leprae* antigen stimulation.

6.1. *In vitro* cytokine production in patients with ENL and LL controls

The *in vitro* cytokine response of PBMCs from patients with ENL and LL controls to *M. leprae* WCS before, during and after treatment was compared. The results are given by mean ± standard error of the mean (SE). SE was chosen since the primary objective of the study was to measure how the mean of the sample is related to the mean of the underlying population. SE takes standard deviation and sample size into account.

The mean production of TNF-α in response to *M. leprae* WCS stimulation was significantly higher (83.6.4 ±18.82 pg/mL) in the culture supernatants of PBMCs from patients with ENL than from LL patient controls (19.4pg/mL ±10.44) before treatment (t_{67} =2.629, P≤0.05). During treatment, the level of TNF-α production decreased to 10.7pg/mL in patients with ENL while it was increased to 36.9pg/mL in LL patient controls. After treatment, TNF-α production was not significantly different between the two groups (Figure 6.6.2).

Patients with ENL reactions had a significantly higher (1361pg/mL ± 309.6) IFN-γ production than the LL patient controls (280.1pg/mL ± 309.6) before treatment ($t_{66=}$ 2.456, P≤ 0.05). However, during treatment, the level of IFN-γ production was significantly decreased in patients with ENL to 304.4 pg/mL ± 119.6 while it was increased in LL patient controls to 1158.0 pg/mL ± 549.2 (t_{62}=2.720, P≤ 0.05). The level of IFN-γ production was similar in these two groups after treatment (Figure 6.6.2).

Although the amount of IL-1β production was similar in patients with ENL and LL controls before and during treatment, patients with ENL had much lower production of IL-1β than LL patient controls after treatment (t_{54}=2.112, P≤ 0.05). On the other hand, higher production of IL-17A was obtained in patients with ENL cases compared to LL patient controls before treatment (t_{48}=3.474.112, P≤ 0.005). Although the *in vitro* production of IL-17A was considerably decreased after treatment in patients with ENL, it still remained high compared to the corresponding figure in LL patient controls (t_{48}=2.098.112, P≤ 0.05) (Figure 6.6.2).

The *in vitro* response of IL-6 production was higher in patients with ENL than in LL controls before treatment (t_{73}=3.853, P≤ 0.005). However, during and after treatment, the *in vitro* IL-6 production was similar in both groups. On the other hand, the level of IL-8 production was found to be higher in patients with ENL than in LL controls throughout the study period (P≤ 0.05) (Figure 6.6.3).

The response of IL-10 to *M. leprae* WCS both in patients with ENL and LL controls was also investigated. IL-10 production was considerably lower in patients with ENL (18.59pg/mL ± 4.05) than in LL patient controls (122.6 pg/mL ± 23.18) before treatment (t_{67}=5.277, P<0.0001). Interestingly, after treatment, the level of IL-10 production was significantly increased to 87.78pg/mL ± 22.49 in patients with ENL while it was substantially decreased to 4.91pg/mL ± 2.160 in LL patient controls and the difference was statistically significant (P ≤ 0.001) (Figure 6.6.3).

In conclusion, the *in vitro* production of the cytokines TNF-α, IFN-γ, IL-17A, IL-6 and IL-8 were higher in patients with ENL than in LL patient controls before treatment while IL-10 was significantly lower in patients with ENL than in LL patient controls at recruitment.

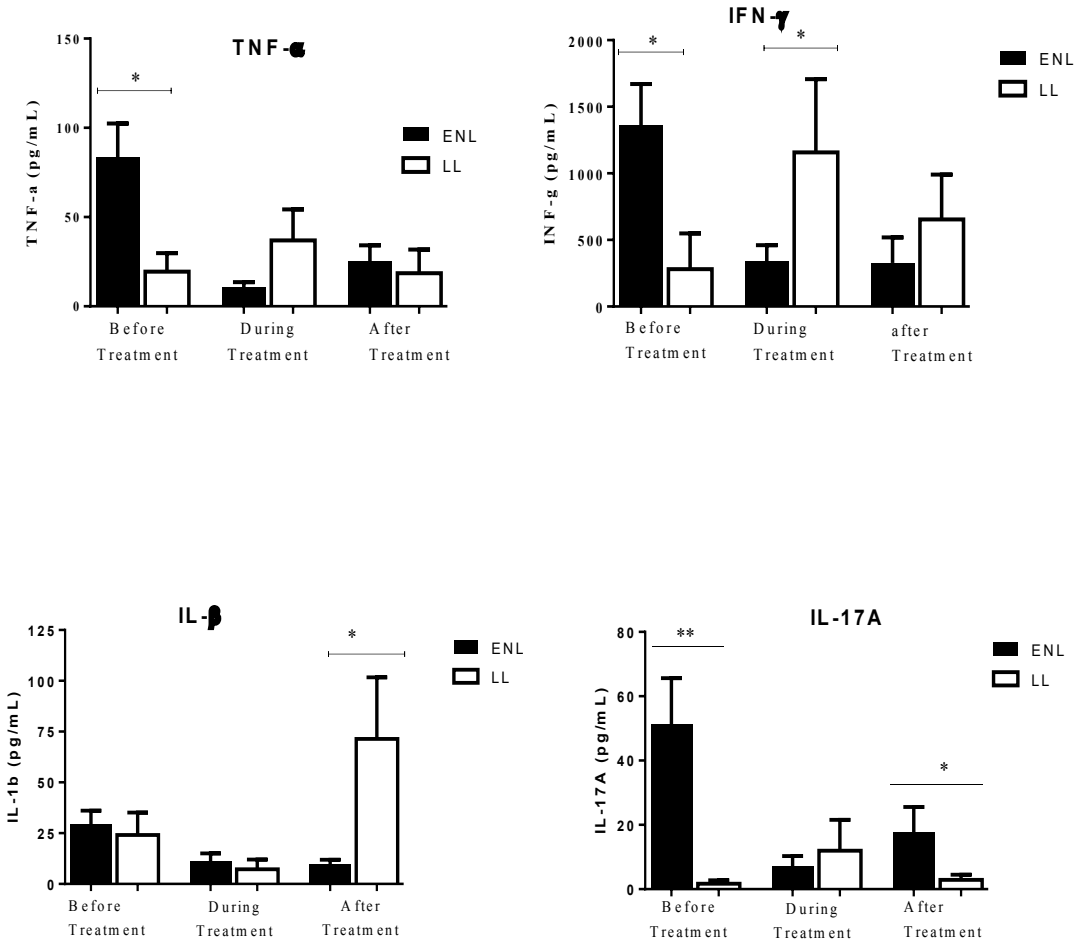


Figure 6.6.2. Comparison of the levels of *in vitro* production of TNF-α, IFN-γ, IL-β and IL-17A in culture supernatants of PBMCs from patients with ENL and LL controls before and after treatment. ENL: n (before) =46, n (during) = 38, n (after) = 35; LL: n (before) =31, n (during) = 26, n (after) =25. Statistical test: unpaired t-test, α=0.05. *$P \leq 0.05$; ** $P \leq 0.005$. Bars graphs show mean ± Standard error of the mean.

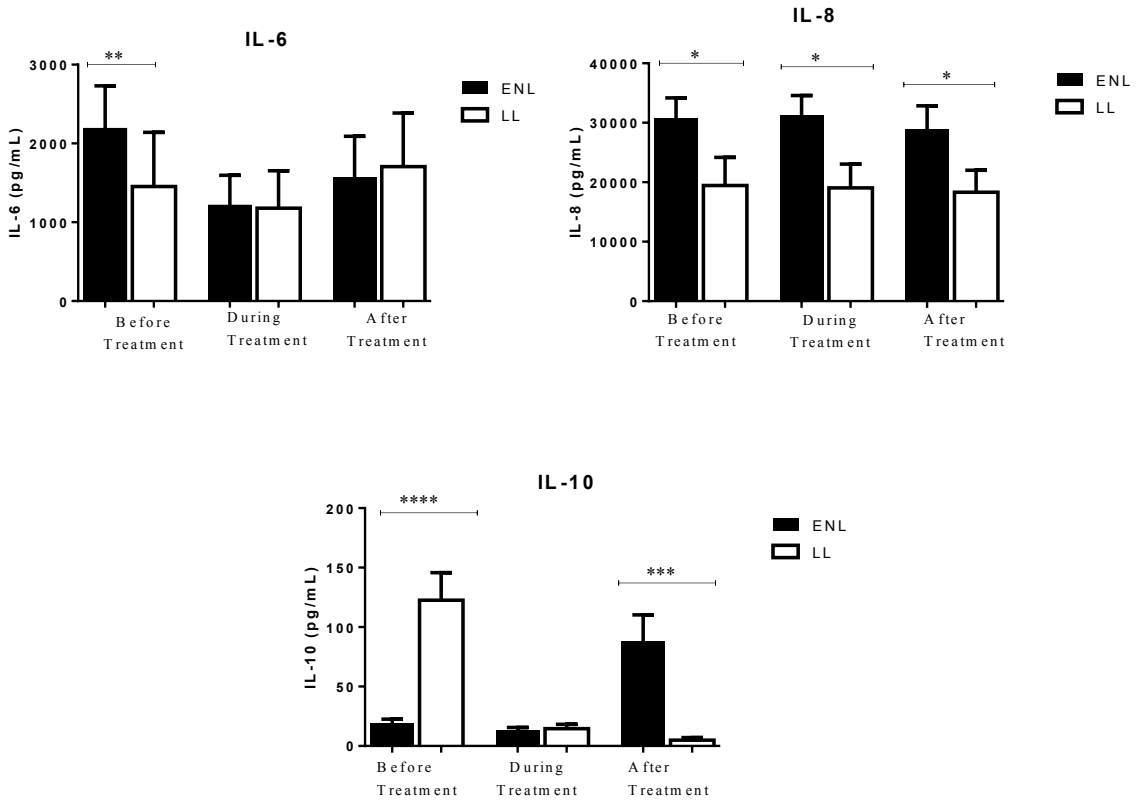
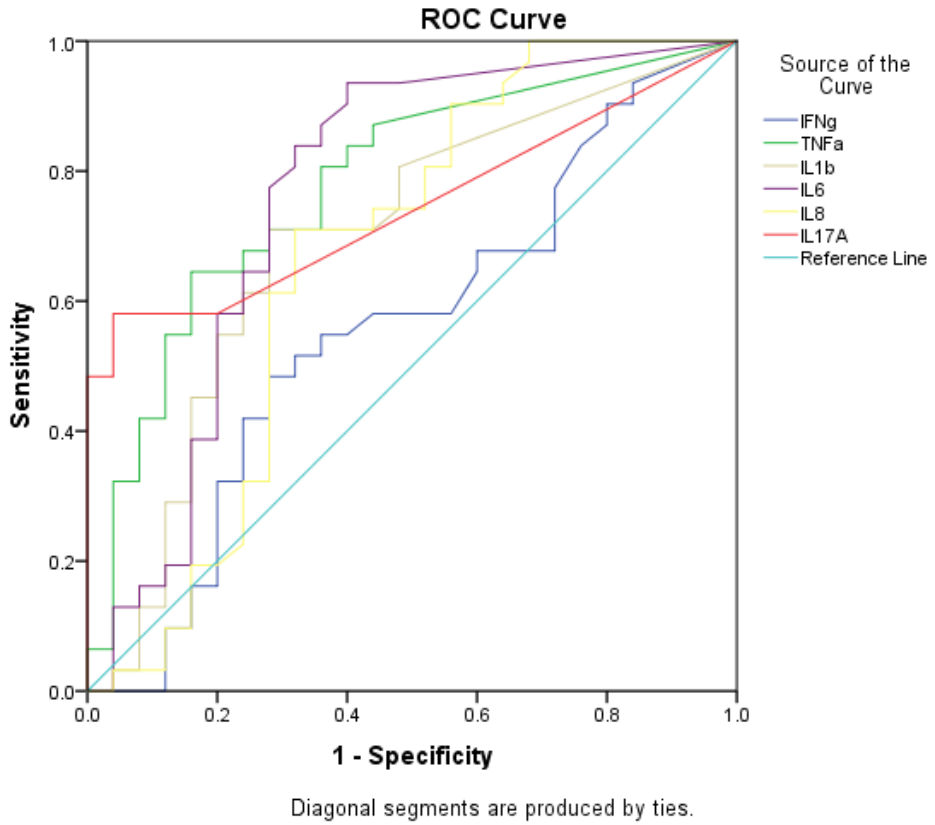


Figure 6.6.3. Comparison of the levels of *in vitro* IL-6, IL-8 and IL-10 production in culture supernatants of PBMCs from patients with ENL and LL controls before and after treatment. n (before) =46, n (during) =38, n (after) =35; LL: n (before) =31, n (during) = 26, n (after) =25. Statistical test: unpaired t-test, α=0.05. *$P \leq 0.05$; **$P \leq 0.005$; ***$P < 0.001$; ****$P < 0.0001$. Bars graphs show mean ± Standard error of the mean.

A receiver operator characteristic (ROC) curve plot for the accuracy of a single cytokine to discriminate between patients with ENL and LL controls was performed. The most accurate cytokines that differentiate between patients with ENL and LL controls were: TNF-α, IL-6, IL-17A, IL-1β, IL-8, IFN-γ and IL-10 with the corresponding of area under the curves (AUCs) of 0.779, 0.763, 0.745, 0.695, 0.667, 0.557 and 0.710 respectively (Figure 6.6.4).



Variables	area under the curve	Std error	Asymptotic sig	Asymptotic 95% confidence interval	
				lower bound	upper bound
TNF-α	0.779	0.063	0.000	0.656	0.903
IL-6	0.763	0.07	0.001	0.625	0.901
IL-17A	0.745	0.066	0.002	0.615	0.874
IL-10	0.710	0.078	0.007	0.556	0.863
IL1-β	0.695	0.074	0.013	0.551	0.839
IL-8	0.667	0.079	0.033	0.513	0.821
IFN-γ	0.557	0.079	0.463	0.402	0.713

Figure 6.6.4. Receiver operator characteristics curves (ROCs) showing the accuracies of individual cytokines in discriminating between patients with ENL and LL controls before treatment. AUC= area under the curve.

6.1.1. Principal component analysis (PCA)

Principal component analysis (PCA) with varimax rotation was conducted to assess the cytokine variables in order to summarize them by reducing the dimension of the dataset into principal components. A loading score statistic was used to select the principal components most associated with the outcome (ENL). The assumption of independent sampling was met. The assumptions of normality, linear relationships between pairs of variables, and the variables being correlated at a moderate level were checked.

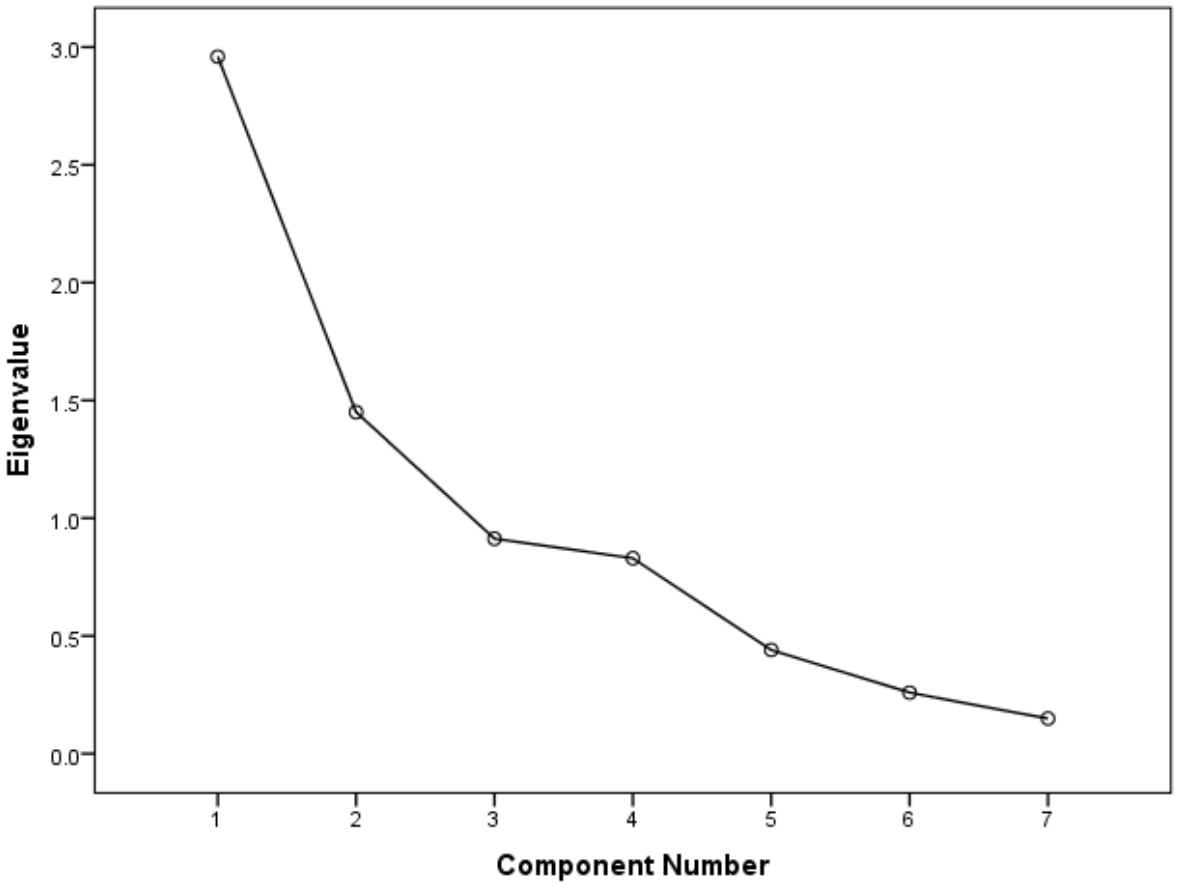


Figure 6.6.5. Scree plot showing the *in vitro* cytokine production in culture supernatants of PBMCs from patients with ENL. A scree plot shows the eigenvalues (y-axis) of the principal components (x-axis) for the data set in descending order and indicates the relative importance of the PCs. Those PCs with eigenvalues <1 account for an increasingly small and insignificant amount of the variance in the data so are not used. In this study the first two PCs have eigenvalues >1 so are the most significant components explaining variability in the data.

The component plot in rotated space (Figure 6.5.6) shows how closely related the cytokines are to each other and to the two components. This plot of the component loadings shows that IL-6, IL-1β and IL-8 all load highly and positively on the first component. IL-10 and TNF-α had near zero on the first component, but load highly on the second component. IL-17A loads moderately on the first component while IFN-γ loads moderately on component 2.

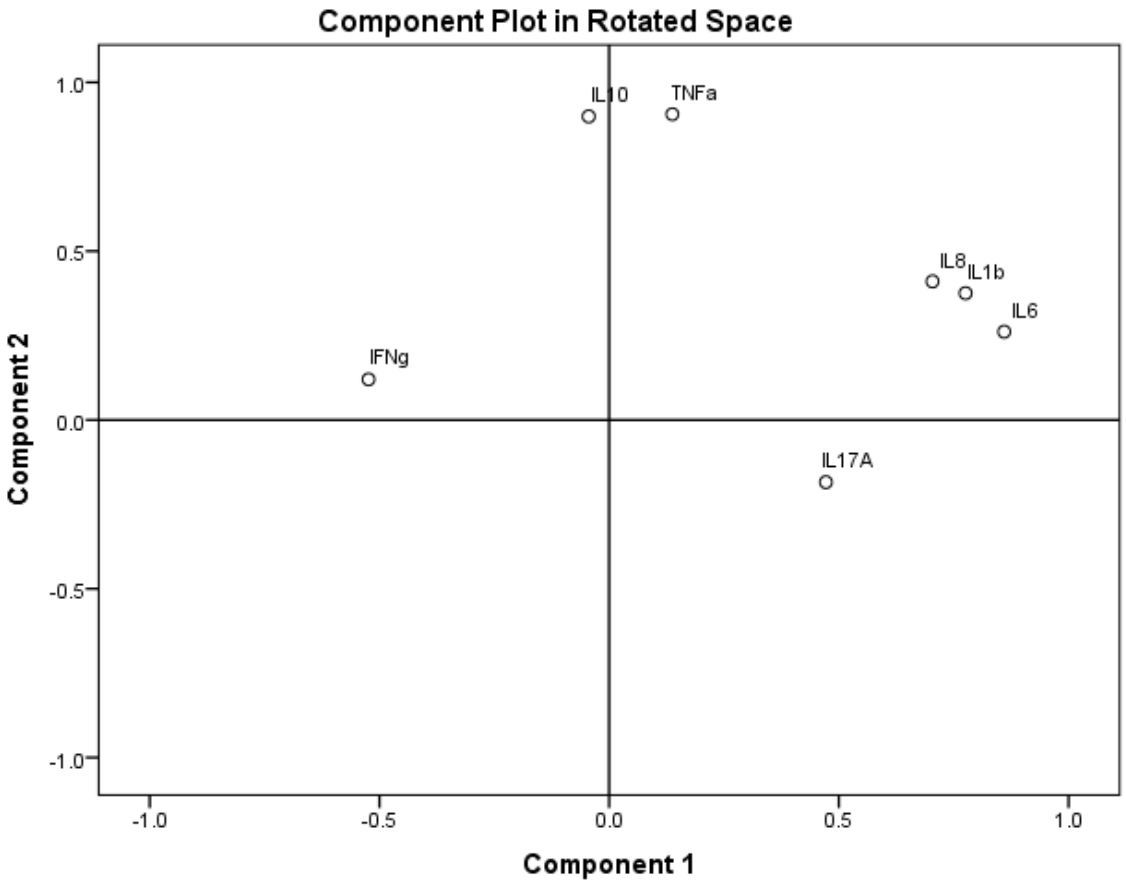


Figure 6.6.6: The component plot in rotated space showing visual representation of the loadings plotted in a 2-dimensional space.

The coefficients shown in table 6.6.1 explain how well each individual cytokine is represented within the clusters. The cytokine levels of IL-6, IL-1β, IL-8 and IL-17 loaded together on the first component, explaining 42.274%of the total variation in the cytokine study. Component 2 is determined by three cytokines: IL-10, TNF-α and IFN-γ explaining 20.714% of the variation.

Table 6.6.1. Individual factor cytokine scores extracted by loading matrix of component solution after Varimax rotation

Cytokines	component 1 loading	component 2 loading
IL-6	0.860	
IL-β	0.776	
IL-8	0.704	
IL-17	0.472	
IL-10		0.899
TNF-α		0.906
IFN-γ		0.120
Total variation explained	42.274%	20.714%
Extraction method: principal component analysis		
Rotation Method: Varimax with Kaiser Normalization		

6.2. *In vitro* cytokine production in ENL before and after treatment

The levels of IFN-γ, IL-17A, TNF-α, IL-6, IL-8, Il-1β and IL-10 production in response to *M. leprae* WCS before, during and after treatment were compared. The in *vitro* TNF-α production in response to *M. leprae* WCS stimulation was considerably higher (83.6pg/mL ± 18.82) before treatment than during treatment (10.7pg/mL±2.79) (t_{34}=3.924, P ≤ 0.001). After treatment, it was slightly increased to 25.4pg/mL±8.88 but was still lower than the amount obtained before treatment (t_{24}=3.397, P ≤ 0.005). Likewise, the *in vitro* production of IFN-γ in response to *M. leprae* WCS was considerably higher before treatment (1361.0pg/mL ±309.6) than during treatment (304.4pg/mL±119.6) (t_{37}=2.957, P ≤ 0.05) and after treatment (328.2pg/mL±190.3) (t_{28}=3.027, P ≤ 0.05) (Figure 6.6.7).

The level of IL-1β and IL-17A production were also found to be higher before treatment than during and after prednisolone treatment of patients with ENL. Although IL-6 production was appreciably decreased during treatment, it was increased after treatment and statistically significant difference was not revealed before or after treatment. Unlike IL-6, the production of IL-8 did not show any significant change before, during and after treatment (Figure 6.5.7). On the other hand, the level of IL-10 production was very low before and during treatment but substantially increased after treatment (Figure 6.6.7).

In summary, the *in vitro* production of IFN-γ, IL-17A, TNF-α and Il-1β to *M. leprae* WCS stimulation were higher before treatment and have shown a significant reduction after treatment indicating the possible association of these proinflammatory cytokines and ENL reaction. On the other hand, the *in vitro* production of IL-10 was noticeably low before treatment and significantly increased after treatment showing its possible regulatory activity. IL-6 and IL-8 did not show significant change before and after treatment of patients with ENL.

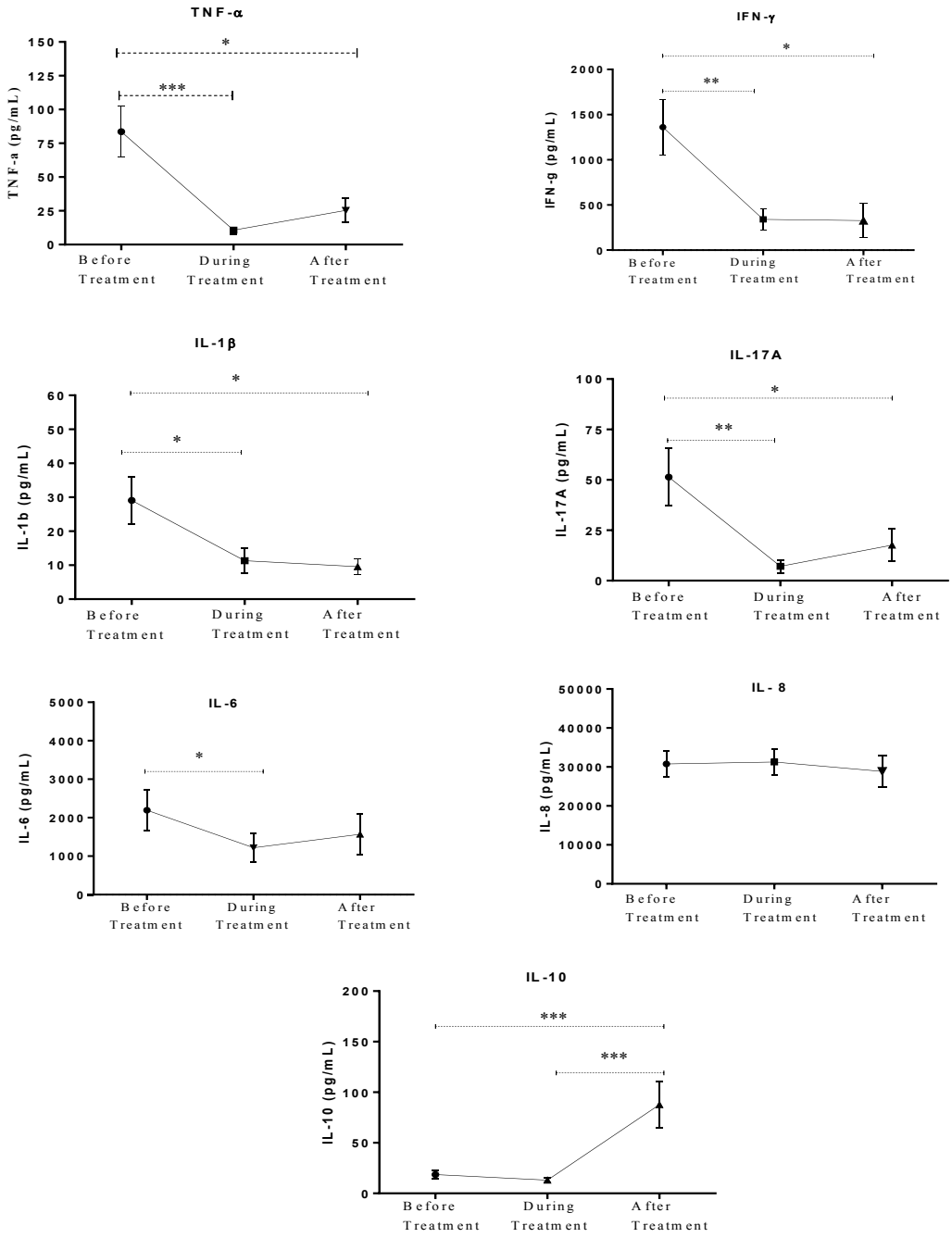


Figure 6.6.7. Comparison of the levels of *in vitro* cytokine production in culture supernatants of PBMCs from patients with ENL before and after treatment. ENL: n (before) = (during) = (after) = 35; LL: n (before) =n (during) = n (after) =25. Statistical test: paired t-test, α=0.05. *$P \leq 0.05$; **$P \leq 0.005$; ***$P < 0.001$; ****$P < 0.0001$. Error bars show mean ± Standard error of the mean.

SECTION 7: CYTOKINE GENE EXPRESSION

To investigate the cytokine gene expression, mRNA was isolated from whole blood and skin biopsies and then reverse transcribed into cDNA. The mRNA copy numbers were quantified on the Corbett Rotor-Gene using real-time PCR assays specific to TNF-α, IFN-γ, IL-β, TGF-β, IL-17A, IL-6, IL-8, IL-10 and HUPO as described in the materials and methods section. The mRNA expression level of FOXP3 in whole blood and biopsy samples was also quantified to supplement the flow cytometry data aimed to measure Tregs. Fold change (FC) was used to compare the levels of these gene expressions in patients with ENL and LL controls as well as among patients with ENL before and after treatment.

7.1. Comparison of cytokine gene expression in the blood samples from patients with ENL and LL controls

The cytokines gene expression in whole blood samples from patients with ENL before and after treatment was compared to LL patient controls. The gene expression levels of TNF-α (FC=3.31) and IFN-γ (FC=2.42) were significantly increased in the blood samples from patients with ENL compared to LL patient controls before treatment ($P \leq 0.005$). However, after treatment, the expression levels of these cytokines were the same in both groups. The gene expression levels of IL-6 (FC=6.01), IL-8 (FC=3.19) and IL-17A (FC=3.58) were considerably high in the blood samples from patients with ENL compared to LL patient controls ($P \leq 0.001$) before treatment but none of them showed a statistically significant difference after treatment (Table 6.7.1).

Although, the fold changes (FC) of mRNA gene expression for IL-1β and TGF-β were slightly increased before treatment in the blood samples from patients with ENL compared to LL patient controls, statistically a significant difference was not revealed. On the other hand, the mRNA gene expression for TGF-β was increased (FC=4.82) in patients with ENL compared to LL patient controls after treatment ($P \leq 0.05$). The level of IL-10 expression was not significantly different in the two groups before and after treatment (Table 6.7.1).

The mRNA expression level of FOXP3 in the blood samples from patients with ENL cases and LL controls did not show a statistically significant difference before

treatment. On the other hand, the gene expression of FOXP3 was evidently increased (FC=2.07) in patients with ENL cases after treatment compared to LL patient controls (Table 6.1). Except IL-10, the result of mRNA gene expression of the other cytokines (TNF-α, IFN-γ, IL-β, TGF-β, IL-17A, IL-6 and IL-8) was found to be consistent with the result of the corresponding *in vitro* cytokine production. Unlike the IL-10 cytokine production in the culture supernatants in response to *M. leprae* WCS, its gene expression in patients with ENL did not reveal statistically significant difference compared to LL patient controls.

Table 6.7.1. Cytokine mRNA expression in the blood samples from patients with ENL and LL controls before and after treatment.

Gene of interest	Before treatment			After treatment		
	$\Delta\Delta C_T$	FC	P- value	$\Delta\Delta C_T$	FC	P- value
TNF-α	-1.73	3.31	0.0047*	0.00	1.00	0.9989
IFN -γ	-1.27	2.42	0.0044*	0.08	0.95	0.8804
IL-1β	-1.23	2.34	0.1085	-0.24	1.18	0.7333
IL-6	-2.59	6.01	0.0003*	-0.99	1.99	0.1167
IL-8	-1.68	3.19	< 0.0001*	-0.81	1.75	0.243
IL-10	0.83	0.56	0.1552	0.28	0.82	0.5882
IL-17A	-1.84	3.58	0.0002*	1.12	0.46	0.0502
TGF-β	-0.07	1.05	0.8724	-2.27	4.82	0.0119*
FOXP3	0.33	0.80	0.6812	-1.05	2.07	0.0083*

n (before) =46, n (during) = (after) = 44; LL: n (before) = (during) = 31, n (after) =28. Statistical test: unpaired t-test, α=0.05.$\Delta\Delta C_T$ = delta delta C_T, FC= fold change, * = significant at *α=0.05*.

7.2. Comparison of cytokine gene expression in skin biopsy samples from patients with ENL and LL controls

The cytokine gene expression in the skin biopsies from patients with ENL before and after treatment was compared to LL patient controls. The expression of mRNA for TNF-α (FC=2.04), IFN-γ (FC=4.01), IL-1β (FC=6.35), IL-6 (FC=5.30) and IL-17A (FC=2.99) were significantly higher ($P \leq 0.005$) in the biopsies from patients with ENL than in the biopsies from LL patient controls before treatment. However, except IL-6 (FC=2.07, $P \leq 0.05$), statistically, a significant difference was not obtained in terms of mRNA gene expression for these cytokines in patients with ENL and LL controls after treatment. The mRNA expression for IL-10 was significantly lower (FC= 0.38) in patients with ENL than in LL patient controls ($P \leq 0.0001$) before treatment. However, after treatment, it was significantly increased in patients with ENL (FC=3.5) compared to LL patient controls and the difference was statistically significant ($P \leq 0.0001$). The mRNA expression for TGF-β and IL-8 in patients with ENL and LL controls did not show statistically significant difference after treatment (Table 6.7.2).

Unlike in the blood sample, the mRNA expression for FOXP3 in tissue samples from patients with ENL was significantly lower (FC =0.35) than in LL patient controls before treatment ($P \leq 0.005$) but statistically a significant difference was not detected between the two groups after treatment (Table 6.7.2). Except for some minor differences, the mRNA expression levels for most of these cytokines showed similar pattern both in blood and tissue samples. The most interesting difference was seen in mRNA expression for IL-1β and FOXP3. No significant difference in expression levels of mRNA for IL-1β was detected in blood samples from patients with ENL and LL controls but substantially a higher mRNA expression for IL-1β was found in tissue samples from patients with ENL than from LL patient controls before treatment. Similarly, the mRNA expression for FOXP3 was not significantly different in the blood samples from patients with ENL cases and LL controls before treatment but was increased by two-fold in patients with ENL after treatment. On the other hand, mRNA expression for FOXP3 in tissue samples from patients with ENL was significantly decreased before treatment compared to LL controls but statistically significant difference was not obtained between the two groups after treatment (Table 6.7.2).

Table 6.7.2. Cytokine mRNA expression in the tissue samples from patients with ENL and LL controls before and after treatment.

Gene of interest	Before treatment			After treatment		
	$\Delta\Delta C_T$	FC	P -value	$\Delta\Delta C_T$	FC	P-value
TNF-α	-1.03	2.04	0.0033*	-0.09	1.07	0.89
IFN -Ɣ	-2.01	4.01	0.0058*	-0.32	1.25	0.5224
IL-1β	-2.67	6.35	0.0006*	-0.24	1.18	0.7333
IL-6	-2.41	5.30	0.0015*	-1.05	2.07	0.0438 *
IL-8	-0.12	1.09	0.8465	-0.71	1.64	0.2333
IL-10	1.41	0.38	< 0.0001*	-1.81	3.50	< 0.0001*
IL-17A	-1.58	2.99	0.0035*	-0.13	1.10	0.281
TGF-β	0.31	0.8	0.4876	-0.63	1.55	0.3598
FOXP3	1.52	0.35	0.0008*	0.23	0.86	0.6845

n (before) =46, n (during) = (after) = 44; LL: n (before) = (during) = 31, n (after) =28. Statistical test: unpaired t-test, α=0.05.$\Delta\Delta C_T$ = delta delta C_T, FC= fold change, * = significant at *α=0.05*.

7.3. Comparison of cytokine gene expression in the blood samples from patients with ENL before and after treatment

The gene expression levels of the above cytokines were also investigated in blood samples from patients with ENL before and after treatment. The ΔΔCT of each pair was obtained by subtracting the ΔΔCT before treatment from ΔΔCT after treatment (ΔΔCT after – ΔΔCT before). Paired t-test was used to compare the two groups at 5 % significance level.

The mRNA expression for IL-8 and TGF-β did not show a statistically significant difference before and after treatment. On the other hand, the gene expression levels of IFN-γ ($P < 0.0001$), IL-6 ($P \leq 0.0001$), IL-1β ($P \leq 0.005$) and TNF- α ($P \leq 0.05$) were significantly decreased in the blood samples from patients with ENL after treatment while it was significantly increased for IL-10 ($P \leq 0.0001$). The mRNA gene expression for FOXP3 in these patients was not significantly different before and after treatment (Table 6.7.3).

Table 6.7.3. Cytokine mRNA expression in the blood samples from ENL before and after treatment.

Cytokine	$\Delta\Delta C_T$	FC	P Value	Gene expression after treatment
TNF-α	1.19	0.44	0.0497	Decreased
IFN -Ɣ	1.61	0.33	< 0.0001	Decreased
IL-1β	1.56	0.34	0.0043	Decreased
IL-6	1.60	0.33	< 0.0001	Decreased
IL-8	0.48	0.72	0.0790	No change
IL-10	-1.32	2.50	< 0.0001	Increased
IL-17A	2.34	0.20	< 0.0001	Decreased
TGF-β	0.24	0.85	0.7160	No change
FOXP3	0.33	0.80	0.5564	No change

n (before) = (during) = (after) = 44; LL: n (before) = (during) = (after) =28.
Statistical test: paired t-test, $\alpha=0.05$. $\Delta\Delta C_T$ = delta delta C_T, FC= fold change, * = significant at $\alpha=0.05$.

7.4. Comparison of cytokine gene expression in the skin biopsy samples from patients with ENL before and after treatment

The cytokine gene expression in the skin biopsy samples from patients with ENL before and after treatment was compared. The mRNA expression levels in biopsy samples for TNF-α (P≤ 0.0001), IFN-γ (P≤ 0.0001), IL-1β (P≤ 0.0005) and IL-17A (P≤ 0.0204) were significantly decreased after treatment. On the other hand, mRNA expression level for IL-10 was considerably increased after treatment (P≤ 0.0001. The mRNA expression for IL-6, IL-8 and TGF-β did not show statistically a significant difference before and after treatment (Table 6.7.4).

Interestingly, unlike the mRNA expression for FOXP3 in the blood samples, the expression in the biopsy samples from patients with ENL was significantly increased after treatment (P≤ 0.0001) which shows the possibility of local immune regulation at the site of reactional lesions in these patients (Table 6.7.4).

Table 6.7.4. Cytokine mRNA expression in skin biopsy samples from patients with ENL before and after treatment.

Cytokine	$\Delta\Delta C_T$	FC	P Value	Gene expression after treatment
TNF-α	2.34	0.2	< 0.0001*	Decreased
IFN -Ɣ	3.03	0.12	< 0.0001*	Decreased
IL-1β	2.39	0.19	0.0005*	Decreased
IL-6	0.31	0.81	0.6451	No change
IL-8	0.16	0.90	0.7617	No change
IL-10	-1.51	2.85	< 0.0001 *	Increased
IL-17A	1.01	0.5	0.0204 *	Decreased
TGF-β	0.09	0.94	0.8337	No change
FoxP3	-3.07	8.4	< 0.0001*	Increased

n (before) = (during) = (after) = 44; LL: n (before) = (during) = (after) =28. Statistical test: paired t-test, α=0.05.$\Delta\Delta C_T$ = delta delta C_T, FC= fold change, * = significant at *α=0.05*.

Table 6.7.5. Summary of the trends in cytokines gene expression and in vitro cytokine production to the response of *M. leprae* stimulation in patients with ENL and LL controls before and after treatment

Cytokines	Before treatment		After treatment	
	ENL	LL	ENL	LL
In vitro cytokine production				
TNF-α	↑	↓	–	–
IL-1β	–	–	↓	↑
IFN-γ	↑	↓	–	–
IL-6	↑	↓	–	–
IL-8	↑	↓	↑	↓
IL-10	↓	↑	↑	↓
IL-17A	↑	↓	↑	↓
Gene expression in blood samples				
TNF-α	↑	↓	–	–
IL-1β	–	–	–	–
IFN-γ	↑	↓	–	–
IL-6	↑	↓	–	–
IL-8	↑	↓	–	–
IL-10	–	–	–	–
IL-17A	↑	↓	–	–
TGF-β	–	–	↑	↓
Gene expression in biopsy samples				
TNF-α	↑	↓	–	–
IL-1β	↑	↓	–	–
IFN-γ	↑	↓	–	–
IL-6	↑	↓	↑	↓
IL-8	–	–	–	–
IL-10	↓	↑	↑	↓
IL-17A	↑	↓	–	–
TGF-β	–	–	–	–

↑= increased, ↓= decreased, – = no change

Table 6.7.6. Summary of the trends in cytokines gene expression and *in vitro* cytokine production to the response of *M. leprae* stimulation in patients with ENL before and after treatment.

In vitro cytokine production	Before treatment	After treatment
TNF-α	↑	↓
IL-1β	↑	↓
IFN-γ	↑	↓
IL-6	–	–
IL-8	–	–
IL-10	↓	↑
IL-17A	↑	↓
Gene expression in blood samples		
TNF-α	↑	↓
IL-1β	↑	↓
IFN-γ	↑	↓
IL-6	↑	↓
IL-8	–	–
IL-10	↓	↑
IL-17A	↑	↓
TGF-β	–	–
Gene expression in biopsy samples		
TNF-α	↑	↓
IL-1β	↑	↓
IFN-γ	↑	↓
IL-6	–	–
IL-8	–	–
IL-10	↓	↑
IL-17A	↑	↓
TGF-β	–	–

↑= increased, ↓= decreased, – = no change

SECTION 8: PLASMA C1Q PRODUCTION AND ITS GENE EXPRESSION

Complement C1q is the first subcomponent of the C1 complex of the classical pathway of complement activation. C1q recognizes and binds to immunoglobulin complexed to antigen and initiates the complement cascade. It has been confirmed that C1q deficiency or abnormality is associated with increased incidence of immune complex diseases such as systemic lupus erythematosus, polymyositis, glomerulonephritis, and Henoch-Schonlein purpura.

In the present study, the circulating C1q in the plasma of patients with ENL and LL controls before and after treatment was quantified using a special C1q-ELISA and its gene expression by quantitative PCR. The gene expression of C1q in the biopsy samples obtained from patients with ENL reactions and LL controls before and after treatment was also determined.

8.1. Plasma C1q production

The circulating C1q plasma was measured by ready-to-use sandwich ELISA. Mean ± standard error of the mean (SE) was used for comparison between cases and controls. Patients with ENL reaction had significantly a lower circulating C1q (11698 ± 618.3) than LL patient controls (21059 ± 2382.0) before treatment ($P \leq 0.001$). However, after treatment, the amount of circulating C1q in both groups did not show statistically a significant difference (Figure 6.8.1).

The circulating C1q in patients with ENL before and after prednisolone treatment was compared. The mean circulating C1q in the plasma from patients with ENL reaction was considerably increased from 11698 pg/mL ±618.3 before treatment to 22287pg/mL ±2154.0 after treatment and the difference was statistically significant ($t_{33}=5.071$, $P < 0.0001$) (Figure 8.1). This finding indicates that patients with ENL reactions do not have genetic defects of C1q since the level of C1q production increased after the ENL reactions subside.

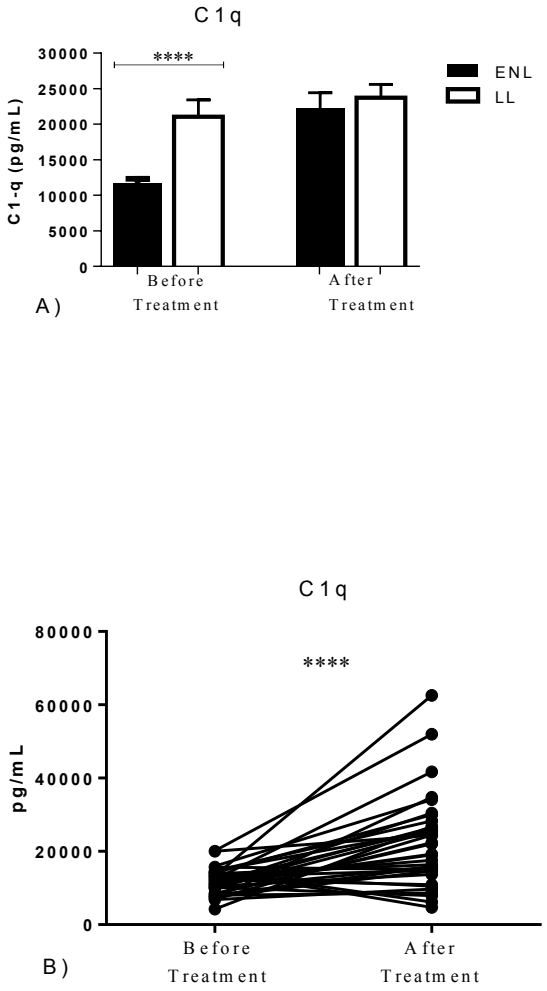


Figure 6.8.1. Plasma circulating C1q levels before and after treatment. A): circulating C1q in the plasma of patients with ENL and LL controls before and after treatment, B): circulating C1q before and after prednisolone treatment of patients with ENL. ENL: n (before) = 46, n (after) = 44; LL: n (before) = 31, n (after) =28. Statistical test: for ENL versus LL we used unpaired t-test and for comparison within ENL paired t-test. **** $P < 0.0001$. Bars graphs show mean ± Standard error of the mean.

8.2. C1q gene expression in blood and skin biopsy samples

C1q is an 18-subunit glycoprotein consisting of 3 subunits: A, B and C which are coded by *C1qA*, *C1qB* and *C1qC* genes respectively. It has been shown that C1q is assembled in a 1:1:1 ratio from these three different subunits. The three human C1q genes are closely located on chromosome 1 and arranged *C1qA-C1qC-C1qB* orders. In this study, the mRNA expression for these three genes in the blood and biopsy samples obtained from patients with ENL and LL controls before and after treatment was quantified.

The gene expression levels for *C1q A*, *B* and *C* in the blood samples from patients with ENL and LL controls were similar before treatment. However, the gene expressions of these three genes significantly increased in blood samples from patients with ENL compared to LL patient controls after treatment (Table 6.8.1). The mRNA expression levels of these genes in biopsy samples from patients with ENL and LL controls were not statistically significantly different before treatment similar to the results obtained from blood samples. After treatment, the expression of *C1qA* was significantly increased while that of *C1qC* was significantly decreased in biopsy samples from patients with ENL compared to in LL patient controls (Table 6.8.1).

Table 6.8.1. C1q mRNA expression in blood and skin biopsy samples from patients with ENL and LL controls before and after treatment.

Sample	gene of interest	Before treatment			After treatment		
		$\Delta\Delta C_T$	FC	P Value	$\Delta\Delta C_T$	FC	P Value
Blood	C1q-A	-0.54	1.45	0.3928	-1.36	2.56	0.0069 *
	C1-B	-0.80	1.74	0.3576	-1.39	2.63	0.0402 *
	C1q-C	-0.58	1.49	0.4044	-2.19	4.55	0.0018 *
Tissue	C1q-A	0.37	0.78	0.7661	-2.69	6.45	0.0231*
	C1-B	0.89	0.54	0.2072	0.56	0.68	0.3713
	C1q-C	1.13	0.46	0.0737	2.90	0.13	< 0.0001 *

ENL: n (before) = (after) = 44; LL: n (before) = (after) =28. Statistical test: paired t-test. $\Delta\Delta C_T$ =delta delta C_T; *statistically significant at α=0.05.

The mRNA expression levels for C1q genes in blood and biopsy samples from patients with ENL before or after treatment were also compared. The expressions of mRNA for all the three genes in blood samples were not statistically significantly different before and after treatment of patients with ENL. Interestingly, the mRNA expression for *C1qA* was remarkably increased after treatment ($P < 0.0001$). On the other hand, the gene expression for *C1qC* was significantly decreased ($P < 0.0001$) after treatment of patients with ENL (Table 6.8.2).

Table 6.8.2. C1q mRNA expression in blood and skin tissue samples from patients with ENL before and after treatment.

Samples	Gene of interest	$\Delta\Delta C_T$	FC	P-value	Gene expression after treatment
Blood	C1q-A	0.66	0.63	0.1116	No change
	C1-B	0.64	0.64	0.3309	No change
	C1q-C	0.43	0.74	0.4178	No change
Tissue	C1q-A	-10.3	1264.26	< 0.0001 *	Increased
	C1-B	0.11	0.92	0.9302	No change
	C1q-C	2.93	0.13	< 0.0001 *	Decreased

ENL: n (before) = (after) = 44; LL: n (before) = (after) =28. Statistical test: paired t-test. $\Delta\Delta C_T$ =delta delta C_T; *statistically significant at α=0.05.

SECTION 9: LEVELS OF ANTI PGL-1, Ag85 AND LAM ANTIBODIES

The levels of anti ND-O-BSA (PGL-1), LAM and Ag85 antibodies were measured by ELISA in the plasma samples of patients with ENL and LL controls before and after treatment. BSA was used as negative control and all tested samples had an OD value less than 0.2 for BSA. An OD value above 1.5 was assigned as high response for PGL-1 and LAM, and 1.0 for Ag85.

The level of anti-PGL-1 antibody was not significantly different in patients with ENL (OD= 1.430 ± 0.1281) and LL controls (1.341 ± 0.1415) before treatment. Similarly, the levels of anti -LAM and anti-Ag85 protein antibody titres were not significantly different in patients with ENL and LL controls before treatment. However, after treatment, patients with ENL had significantly lower anti-PGL-1 antibody titre (1.183 ± 0.1333) than LL controls (2.091 ± 0.1081) ($p < 0.0001$). After treatment, anti -LAM and anti-Ag85 protein antibody titres were lower in patients with ENL than in LL controls ($p < 0.0001$). Interestingly, patients with acute ENL had significantly higher anti-PGL-1 antibody titre than chronic cases before and after treatment (Figure 6.8.1). After treatment, only anti-PGL-1 antibody titre was significantly reduced in patients with ENL while anti-LAM and Ag85 did not change. (Figure 6.9.2).

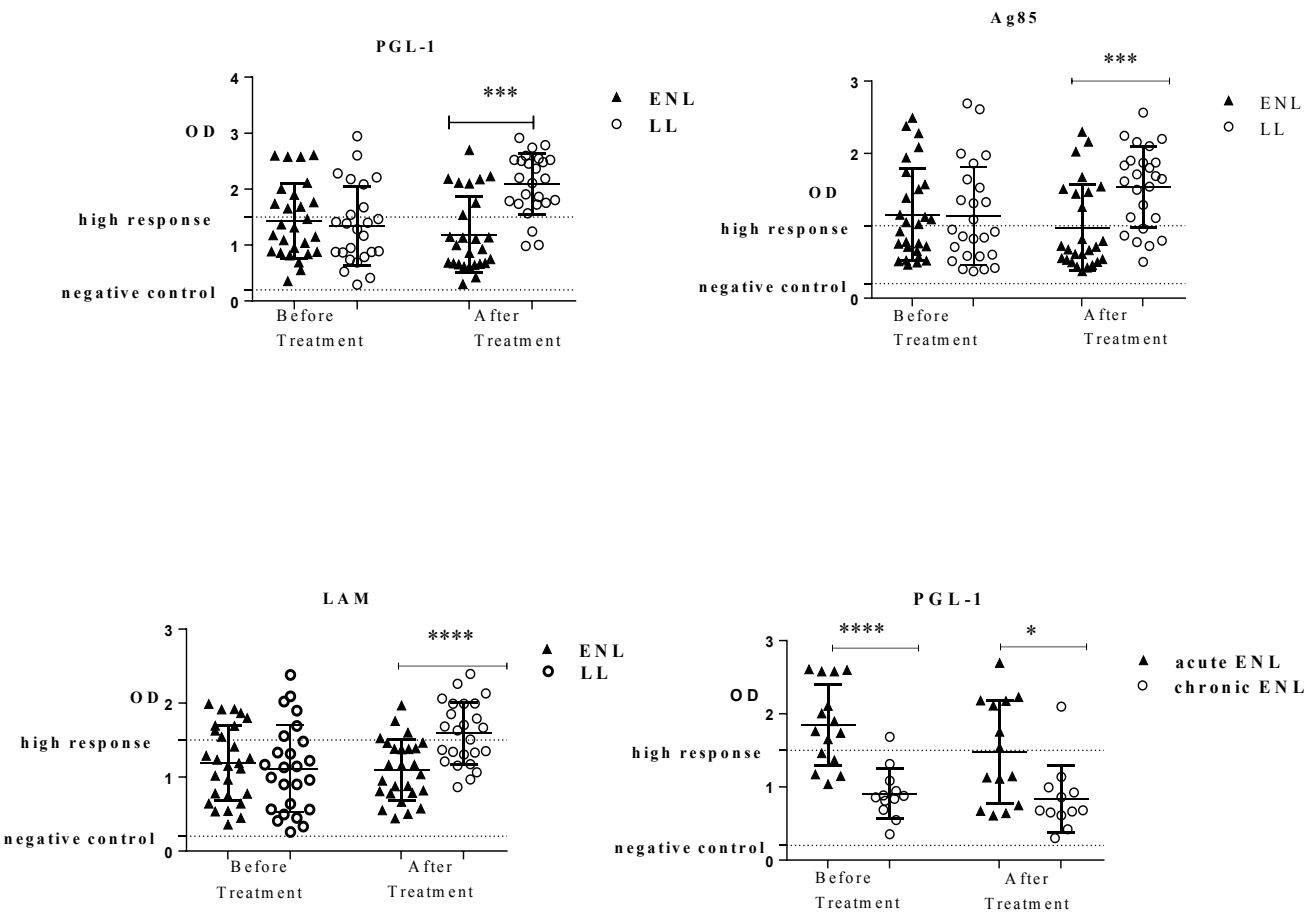
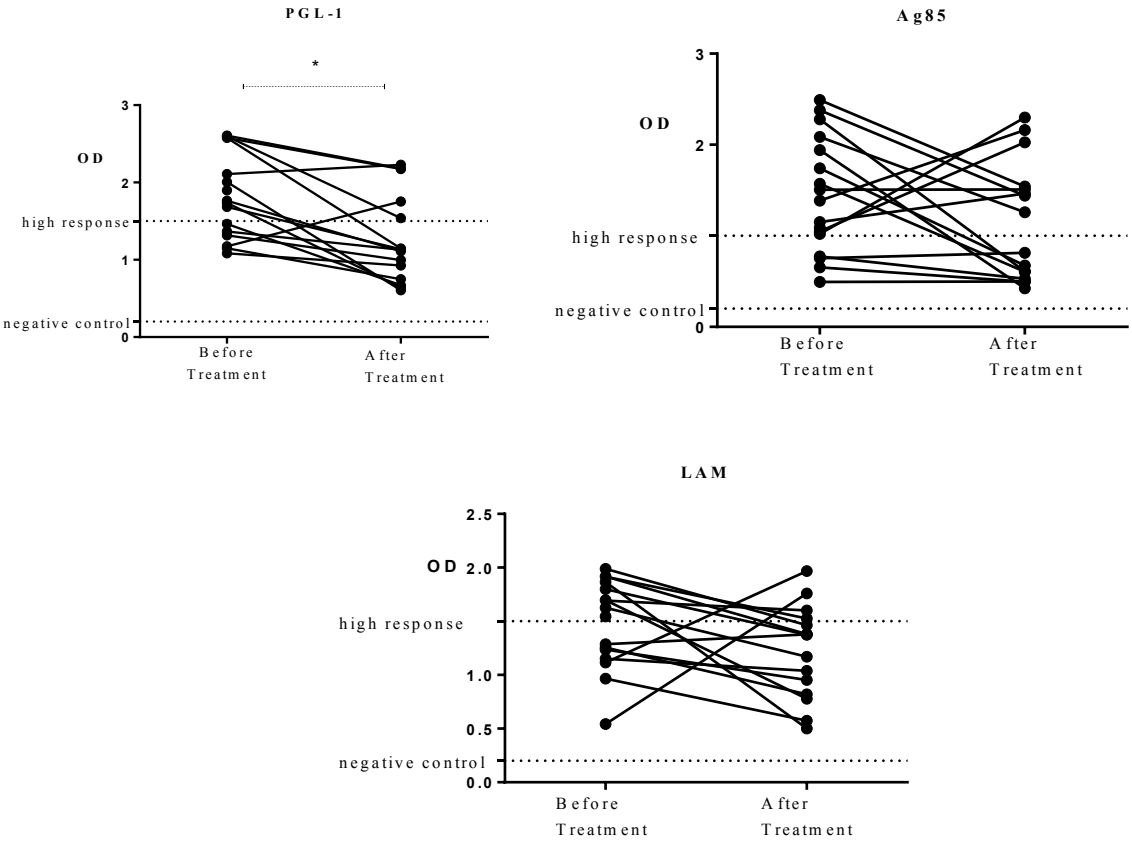


Figure 6.9.1. Levels of anti PGL-1, LAM and Ag85 antibody titres of patients with ENL and LL controls before and after treatment. Values above 1.5 OD for PGL-1 and LAM and above 1.0 OD for Ag85 show high response and values below 0.2 OD show a negative response. ENL: n (before) = n (after) =30; LL: n (before) = (after) =22. OD=optical density, PGL-1= phenolic glycolipid-1, LAM=Lipoarabinomannan, Ag85= Antigen 85 protein. *= *P≤ 0.05*, ***= *P≤ 0.001*, ****= *P<0.0001*. Error bars show median ± interquartile range.



*= P≤ 0.05

Figure 6.9.2. Levels of anti PGL-1, LAM and Ag85 titres in patients with ENL before and after treatment. Values above 1.5 OD for PGL-1 and LAM and above 1 OD for Ag85 show high response and values below 0.2 OD show a negative response. ENL: n (before) = n (after) =30. OD=optical density, PGL-1= phenolic glycolipid-1, LAM=Lipoarabinomannan, Ag85= Antigen 85 protein. *= *P≤ 0.05.*

CHAPTER 7: DISCUSSION

Introduction

This work is the first study involving a large number of patients with ENL and LL controls with follow-up for 28 weeks, which obtained clinical data and biological samples at three time points from each patient during this follow-up. Well- formulated case definitions and controls were applied to get clear representative patients and hence, the results can be extrapolated to the underlying population. Several parameters which included clinical assessment to sophisticated laboratory investigations were also measured. Several immune profiles both in blood and skin biopsy samples from gene expression to protein production levels were investigated. We measured several subsets of T-cells such as the $CD4^+/CD8^+$ balance, regulatory T-cells, activated T-cells, memory T-cell subtypes. More accurate markers were used to measure the frequency of regulatory T-cells. The proportion of mature B-cells and memory B-cell subtypes were also measured. For the first time memory T and B cells in leprosy are presented which we believe it will open new insights for leprosy research. For the first time the circulating complement, C1q in the plasma and its gene expression in the blood and skin biopsy samples of patients with ENL and LL controls were described.

We also determined the *in vitro* cytokine response of PBMCs from patients with ENL and LL controls to *M. leprae* whole cell sonicate. We included several pro-inflammatory cytokines (IFN-γ, IL-17A, TNF-α, IL-6, IL-8, and IL-1β) and regulatory cytokines (IL-10 and TGF- β). The gene expression levels of these cytokines were measured in blood as well as in skin biopsy samples of each patient. The levels of anti ND-O-BSA (PGL-1), Ag85 protein and LAM antibodies were measured by ELISA in the plasma samples of patients with ENL and LL controls before and after treatment. The response of PBMCs from patients with ENL and LL controls by the analysis of IFN-γ and IL-10 responses to *M. leprae* before and after depletion of $CD25^+$ cells were also measured and confirmed that Tregs are not functionally impaired in ENL patients (data not included in this thesis).

SECTION 1. SOCIODEMOGRAPHIC AND CLINICAL CHARACTERISTICS

Some of the key findings of socio-demographic and clinical characteristics of the study subjects are discussed in the flowing sections.

Patient recruitment: In this study, a very stringent inclusion criteria were applied. We excluded nearly half of patients with ENL reactions (36 ENL cases) from the study after they have passed the screening by nurses and physicians. One of the main inclusion criteria for patients with ENL was no previous treatment with any corticosteroid or completed corticosteroid treatment of 15 days or longer. The excluded patients were individuals who disclosed that they had prednisolone tablets obtained privately from pharmacies without a prescription or through a prescription at local health centres and district primary hospitals. They had denied that they had had prednisolone because they were worried that if they disclosed the information to the diagnosing physician or nurse they would not get good treatment. The clinical coordinator, who was in charge of consenting the study patients was not involved in treatment. He spent a longer time in approaching patients than the diagnosing physician or nurses did, and was able to get the actual information from patients through discussion. It is important to mention that one has to be conscious of this issue when consenting patients with ENL and should not only depend on the information obtained about the patient through the diagnosing physicians or nurses for the reasons mentioned above. The several contradicting research reports on the immunology of ENL could be partially due to the lack of stringent inclusion and exclusion criteria and the strategies used for consenting patients. In most studies, patients are consented by the diagnosing and treating physician or nurse which may affect the quality of information obtained from patients. Therefore, it is advisable that the diagnosing (treating) physicians or nurses should not be involved for consenting patients for research purposes.

Socio-demographic data: In this study, we excluded patients under 18 years and above 65 years old. The number of male patients with ENL recruited to the study was as twice the number of female patients and it was higher than in the report by (Walker et al., 2015). Walker et al. reported that the number of male and female patients with ENL was nearly equal (male: female, 1.1:1). These differences could be attributed to

several factors such as the case definitions used, the study period, the study design and the sample size. However, five-year retrospective data (2008-2013) in the same area have shown the number of male to female ratio to be 1.7:1 which is similar to our finding. In our study, the median age for male and female patients with ENL was 28.0 and 26.7 years respectively. Both male and female patients with ENL were relatively older than the LL patient controls (median age: male=26 years, female =21 years). The slight difference in median age between the two groups could be explained by the chronological order of the disease. Patients usually develop ENL reaction after having either LL or BL clinical forms for some time during the course of the disease. Interestingly, the age range of females in both groups was relatively narrow (18-35 years) compared to males (18-60 years) indicating that either younger females are more likely have access to health institutions for various reasons than older females even in low-income countries where health facilities are relatively inadequate (WHO, 2009) or that ENL is relatively common among younger females of child bearing age due to various biological reasons (Duncan and Pearson, 1984, Lockwood and Sinha, 1999, Motta et al., 2012a).

Acute, recurrent and chronic ENL: Our data confirm that many individuals have chronic ENL (39%). This implies that these patients require corticosteroid treatment for extended periods. High doses of corticosteroids may be required for prolonged periods. However, high doses of corticosteroids do not always control the inflammation and also pose life-threatening risks for patients (Girdhar, 1990, Walker et al., 2007). Chronic ENL cases also continue to be a burden in referral hospitals in these resource poor settings where the disease is prevalent. A study in rural India has shown that families with an ENL case are incurred coasts of more than 40% of total household income compared to families without ENL case due to out of pocket expenditure for treatment-seeking (direct costs) and loss of income resulting from reduced productivity (earning potential) of household members (indirect costs). This implies that households affected by ENL face a significant economic burden and are at risk of being pushed further into poverty (Chandler et al., 2015).

Skin lesions morphology, pain symptoms and location: In this study, more diverse cutaneous manifestations of ENL were documented than used in ENL case definition. Although nodular lesions were present in more than 95% of the cases, it is worth

mentioning that looking at a wider differential diagnosis to other lesions during clinical assessments for ENL. Pain was reported in 98% of the patients and was the most common symptom indicating the painful nature of ENL. Most patients had skin pain (80.4%), nerve pain (73.9%), joint pain (71.7%) and bone pain (69.2%). The most frequent site of pain due to ENL in our study was the skin which is explained by the fact that 95% of patients with ENL cases had skin lesions. Our finding is in agreement with the report of Walker et al (2015). Bone pain was reported in two-thirds of our study patients which is higher than the report of Walker et al (2015). The difference between the two studies are likely to be due to the methodological differences employed.

Other symptoms of ENL: Nerve function impairment (NFI) was reported in 65% of our study patients, which was higher than the 51.3% NFI in six countries as reported by Walker et al (2015). Among the 65% of ENL patients who reported NFI, 80% had old NFI and hence, the majority of patients with ENL studied were at high risk of developing a permanent disability. A study by Santos Santos et al. (2013) in northern Brazil had identified NFI and leprosy reactions as the main risk factors associated with the development of disability in leprosy patients. The same authors reported that having NFI was strongly associated with the development of physical disability in children under 15 years of age (Santos et al., 2015). In this study, 50% of patients with ENL had WHO disability grade-1 (G1D) while 4.3% had Grade- 2 disability (G2D). The proportion of grade 2 disability was lower than the national figure for Ethiopia (10.2%) in 2014 (Baye 2015). The lower proportion of G2D in our study can be explained by either the failure of correctly classifying into G1D and G2D or patients with G2D were excluded by chance at enrolment.

Histopathological findings: Paraffin- embedded sections of skin biopsy samples from ENL and LL lesions were graded by a histopathologist. Neutrophil infiltration was noted in 58.8% of patients with ENL compared to 14.3% in LL controls histologically before treatment. Hussain et al. (1995) has reported the absence of neutrophil infiltration in 36% of ENL skin lesions in Pakistani patients despite classical signs and symptoms of ENL. Similarly, a cross-sectional study on the histological features of leprosy reactions in Indian patients by Sarita et al. (2013) showed that 43% ENL skin lesions had no histological evidence of neutrophil infiltration. The reports

of these two studies are in agreement with our finding. Contrary to our reports, previous studies by (Ridley et al., 1981, Jayalakshmi et al., 1995, Adhe et al., 2012a), reported the findings of neutrophil infiltration in all ENL lesions. The varying reports of neutrophil infiltration in ENL lesions could be attributed to several factors. For example, Aldhe et al (2012) investigated the presence of cellular neutrophil infiltration on previously histologically confirmed ENL cases. These authors mentioned that diagnosis of ENL was based on clinico-pathological observations, which means that in their case definition of ENL, they already included the presence of neutrophils in histology. The differences in findings could be also due to a delay between the onset of reaction and the timing of obtaining the biopsy in those without neutrophilic infiltrates, as dermal oedema may be missed in older reactional lesions. Discordance between pathologists and standard operating procedures (SOPs) of slide preparation are also potential areas that should be further investigated to evaluate impact on the findings of neutrophil infiltration in tissue sections. It is important to mention that even though previous reports suggested that vasculitis is part of the ENL reaction commonly seen in Indian patients (Sehgal et al., 1986a), only 5(14.7%) of our patients had evidence of vasculitis. Similar observations have been made by the following authors (Adhe et al., 2012b, Sarita et al., 2013).

SECTION 2: REGULATORY T-CELLS

To describe the relative frequency of Tregs in the PBMCs from patients with ENL and LL controls, the markers $CD3^+CD4^+CD25^+FoxP3^+CD^{25+}CD127^{-/lo}$ were used for the definition of $CD4^+$Tregs and $CD3^+CD8^+CD25^+FoxP3^+CD^{25+}CD127^{-/lo}$ for $CD8^+$ Tregs. The relative frequency of these Tregs before, during and after prednisolone treatment of patients with ENL to see the dynamics of T-cell regulation in association with prednisolone treatment were compared. We have shown not only the percentage of Tregs but also the relative balance between $CD4^+$ and $CD8^+$ T-cells, in terms of the expression of CD25 and FoxP3 on $CD4^+$ and $CD8^+$ T-cells.

$CD4^+$ and $CD8^+$ T- lymphocytes (ENL versus LL): The results indicated that patients with ENL had a significantly higher median percentage of $CD4^+$ T-cells and lower $CD8^+$ T-cells before treatment compared to LL controls. Patients with ENL had a higher CD4/CD8 ratio (2.3:1) compared to LL controls (1.4:1). Prednisolone treatment significantly reduced the percentage of $CD4^+$ T-cells in patients with ENL implying that prednisolone could suppress $CD4^+$ T-cells to resolve the inflammation. Other studies elsewhere have shown that prednisolone treatment promotes an immunological state that favours immune regulation rather than inflammation through regulation of $CD4^+$ T-cell proliferation (Luther et al., 2009). Interestingly, even though patients with ENL had a higher percentage of $CD4^+$ and a lower percentage of $CD8^+$ T-cells before treatment, the percentage of these T-cells are within the normal range for Ethiopian population (Tsegaye et al., 2003). However, the CD4/CD8 ratio obtained in our study for patients with ENL was higher than the reference value for apparently healthy Ethiopian adults (1.5:1). At the same time, the $CD4^+/CD8^+$ ratio of LL patient controls was slightly lower than the normal value. Hence, it is logical to conclude that rather than the actual percentage of $CD4^+$ and $CD8^+$ T cells, the balance between the two T-cell subtypes is indeed associated with ENL reactions. Therefore, the higher the $CD4^+/CD8^+$ ratio the greater is the risk of developing ENL reactions. Increased $CD4^+$ counts and $CD4^+/CD8^+$ T-cells ratio in 11 Indian ENL patients has been reported by Hussain et al. (2015) which is in agreement with the present finding. However, they did not look at the status of $CD4^+$ T-cells count and $CD4^+/CD8^+$ ratio during and after treatment unlike the present study. Similar results have been reported by several studies (Modlin et al., 1983, Bach et al., 1983, Wallach et al., 1982, Mshana et al.,

1983, Narayanan et al., 1984). Lymphocyte imbalance ($CD4^+$and $CD8^+$) has also been indicated in the progression of many other diseases elsewhere (Pichler et al., 2009, Lu et al., 2015).

However, in addition to the $CD4^+/CD8^+$ T-cells ratio, other factors such as the expression pattern of costimulatory molecules on T-cells (CD28) and on antigen presenting cells (CD80/86) in patients with ENL need to be explored. Abnormal T-cell co-stimulation and T-cell senescence has been indicated in the expansion of effector memory T-cells and thought to facilitate the breakdown of tolerance in inflammatory diseases such as anti-neutrophil cytoplasmic autoantibodies (ANCA)-associated vasculitis (Wilde,et al 2010). In ANCA-associated vasculitis, CD28 was found to be downregulated on circulating and lesional $CD4^+$ T- cells (Moosing et al 1998) and $CD4^+CD28^-$ T-cells have been described as the major source of pro-inflammatory cytokines (particularly IFN-γ and TNF-α) in Wegener's Granulomatosis (Komocsi et al 2002). These authors have also shown that the severity of the disease was positively correlated with the increased proportion of $CD4^+CD28^-$ T-cells. We found that patients with ENL had increased production of TNF-α and IFN-γ. However, the source/s of these pro-inflammatory cytokines has not been addressed in this study. Therefore, it is important to investigate the sources of these cytokines in patients with ENL.

$CD4^+$ and $CD8^+$ T-cells in patients with ENL before and after treatment: The median percentage of $CD4^+$ T-cells was significantly decreased during and after prednisolone treatment. On other hand, the median percentage of $CD8^+$ T-cells was significantly increased following prednisolone treatment unlike $CD4^+$ T-cells. Interestingly, the $CD4^+/CD8^+$ T-cells ratio was significantly deceased after prednisolone treatment implying the role of prednisolone in re-establishing the immune homeostasis by downregulating excessive $CD4^+$ T-cell activation. Studies have shown that prednisolone reduces $CD4^+$ T-cells (Shevach, 2009, Kasang et al., 2016). A reduction of $CD4^+$ to $CD8^+$ T cells ratio in patients with rheumatoid arthritis when as little as 2.5mg of prednisone was administered every 6hrs has been reported (Hepburn and Slade, 1987). Mshana et al. (1982) were the first to hypothesize that ENL is precipitated by an imbalance of T-lymphocyte subpopulations. According to this hypothesis, ENL has two phases: initiation, due to an imbalance in T-cell subpopulations with decreased suppressor cells and perpetuation. Hence, the finding

of the imbalance of T-lymphocyte subpopulations ($CD4^+$/ $CD8^+$ ratio) in this study would support the initiation of ENL reaction in patients with lepromatous leprosy. The initiation of ENL reaction by hyper activation of T-cells further explained by the findings of the all of the viable bacterial load associated with release of soluble antigenic material at the site of bacterial degranulation in ENL patients compared to the intact bacteria observed in non-reactional LL patients (Rees et al., 1965, Lahiri et al., 2008b). Hence, it seems that unlike in the LL patients, macrophages in ENL patients are activated and process the bacteria and hence fragmented and granular bacterial deposits seen in these patients. The activation of macrophages by T-cells (most likely through IFN-ϒ secretion) may produce inflammatory cytokines such as TNF-α and IL-1β (Arango Duque and Descoteaux, 2014) which could amplify the immune hyperactivation and hence tissue damage in ENL patients.

CD25 and FoxP3 expression on CD4 and CD8 T-cells (ENL versus LL): This study has shown, that the level of CD25 expression on $CD4^+$ T-cells was not significantly different in patients with ENL and LL controls before treatment but the relative expression of $CD8^+CD25^+$ was lower in patients with ENL than in LL patient controls. This could be explained by the fact that CD25 is not only expressed on Tregs but also on activated T-cells as previously described (Corthay, 2009). Hence, it is not appropriate to compare the level of phenotypic expression of CD25 on either $CD4^+$ or $CD8^+$ T-cells in patients with ENL and LL controls since the expression of CD25 could have different implications in these groups: activation in patients with ENL and regulation in non-reactional LL patients.

Although transient FoxP3 expression in *ex-vivo* activated human T-cells has been reported by Wang et al. (2007), it remains as a good candidate marker for Tregs. In this study, for immunophenotyping of T-cell subtypes, unstimulated PBMCs were used and hence our FoxP3 result is less likely to be over represented. The stimulation of $CD4^+CD25^-$ human T-cells have shown to generate $CD4^+CD25^+$ T cells which can also express FoxP3 (R.Walker et al., 2003). The present result shows that the percentage of $CD4^+FoxP3^+$ T-cells in the PBMCs from patients with LL controls was more than twice the percentage of $CD4^+FoxP3^+$ T-cells in the PBMCs from patients with ENL before treatment. However, a similar percentage of $CD4^+FoxP3^+$ was obtained in both groups after treatment. On the other hand, the expression of FoxP3

in $CD8^+$ T-cells was not significantly different in either groups before or after treatment. To obtain a more characterized FoxP3 population that has a regulatory property, CD127 was used in conjunction with CD25 as an additional marker as previously described by Liu et al. (2006). Therefore, the Tregs described here are better characterized than previous reports.

CD25 and FoxP3 expression in patients with ENL before and after treatment: In our study, the expression of CD25 by $CD4^+$ and $CD8^+$ T-cells was not significantly different before and after prednisolone treatment. This can be explained by the fact that CD25 can be expressed by activated as well as regulatory T-cells as previously described (R.Walker et al., 2003). Therefore, although the CD25 expression in $CD4^+$ or $CD8^+$ T-cells before and after treatment is comparable, it may not have the same role i.e. it could play with activation role before treatment and a regulatory role after treatment in the same patient.

Interestingly, the expression of FoxP3 in $CD4^+$ T-cells was significantly increased after prednisolone treatment. Thus, unlike the expression of CD25 in $CD4^+$ T-cells, prednisolone upregulates the expression of FoxP3 in $CD4^+$ T-cells and hence increases tolerance through immune suppression (Luther et al., 2009, Shevach, 2009). On the other hand, the expression of FoxP3 in $CD8^+$ T-cells was not significantly different before and after prednisolone treatment. Hence, it appears that prednisolone does not affect the expression of FoxP3 in $CD8^+$ T-cells.

$CD4^+$ and $CD8^+$ Tregs (ENL versus LL): in this study, the median percentage of $CD4^+$ Tregs in patients with ENL was significantly lower (1.7%) than in LL controls (3.8%) before treatment. In addition, after treatment, the percentage of $CD4^+$ Tregs in patients with ENL was increased to 3.2% while in LL controls it was decreased to 2.5%. Tregs are the least studied T-cells in patients with ENL. There are only three cross-sectional studies by the same authors which had reported the reduced frequency of Tregs in patients with ENL compared to other clinical forms of the spectrum. The study by Attia et al (2010) has reported 1.2% of Tregs in the whole blood of patients with ENL compared to 2.8% in LL controls although the study was included only 6 patients with ENL and 8 LL controls. Another study by the same group in 2014 reported 1.15% and 2.8% of Tregs in the whole blood of 6 patients with ENL and 11 non-reactional LL controls respectively. Although these two studies are in agreement

with the findings here, they reported slightly lower percentages of Tregs in both groups than we found. There are several differences between the study of Attia et al and our study. Firstly, Attia et al included only 6 patients with ENL in both studies and the sample size was too small to draw a valid conclusion. They also obtained blood samples only once and they did not test the trend of Tregs at different time points. Secondly, case definitions for ENL were not mentioned and therefore, it is not clear how patients with ENL were recruited to the study. Thirdly, they used $CD4^{+}CD25^{high}$ $FoxP3^{+}$ as Treg markers. However, a consensus on the thresholds of CD25 expression needed to delineate Treg cells within the $CD25^{high}$ population is difficult to attain, and variations in FoxP3 expression within the $CD25^{high}$ population have been observed even in healthy individuals (Miyara et al., 2009). Hence, the definition of CD25 high and low is highly subjective which could lead to biased reports. In addition, healthy individuals and other clinical forms of leprosy such as TT and T1R have also been used by many authors as a basis for comparison with ENL which leads to discordant results. Only patients with either BL or LL will develop ENL reactions. However, not all patients with BL or LL develop ENL. Hence, for immunological studies either non-reactional BL or LL patients are the appropriate controls for ENL patients.

On the other hand, in this study, the median percentage of $CD8^{+}$ Tregs was not significantly different in patients with ENL and LL controls before or after treatment. Hence, it appears that the development of ENL in patients with lepromatous leprosy is mainly associated with the decreasing frequency of $CD4^{+}$ Tregs being unable to balance the excessive immune activation via $CD4^{+}$ T-cells. Studies have shown that Tregs inhibit naïve $CD4^{+}$ T-cell proliferation and differentiation and hence, downregulate immune activation (Shevach, 2009). Similar findings have also been reported elsewhere in immune-complex mediated autoimmune diseases such as Wegener's granulomatosis (WG) (Morgan et al., 2010) and Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (Marinaki et al., 2005, Wilde et al., 2010).

$CD4^{+}$ and $CD8^{+}$ Tregs in patients with ENL before and after treatment: Our study is the first to present longitudinal data on the frequency of circulating $CD4^{+}$ and $CD8^{+}$ Tregs at different time points in patients with ENL. Furthermore, unlike previous

cross-sectional studies, we used more refined Treg defining markers ($CD3^+CD4^+$ ($CD8^+$) $CD25^+FoxP3^+CD127^{-/lo}$).

Untreated patients with ENL cases had significantly lower percentage of $CD4^+$Tregs which was considerably increased after prednisolone treatment. On the other hand, the frequency of $CD8^+$ Tregs did not show any significant difference before and after prednisolone treatment. Thus, our finding indicates that $CD4^+$ Tregs are associated with ENL reactions but not $CD8^+$ Tregs. Comparison of our finding with previous studies was not possible since our study is the first longitudinal study comparing the dynamics of Tregs at different time points in individual patients with ENL. However, similar findings have been reported in several independent studies elsewhere in autoimmune diseases. Nevertheless, it is not reasonable to make comparisons with autoimmune diseases since the aetiology of the ENL is different from that of autoimmune diseases. Studies in infection models described the significant immunosuppressive activity of Tregs for antigen-specific T-cells. Tregs are considered critical for suppressing immune responses to self-antigens and preventing autoimmunity and for regulating immunity to foreign antigens, especially those derived from pathogens that establish persistent infections (Belkaid and Rouse, 2005).

It has also been shown that Tregs may protect from non-specific memory T-cell activation and potential tissue damage (Arram et al., 2014). Hence, the reduced frequency of $CD4^+$ Tregs and the increased $CD4^+/CD8^+$ T-cells ratio in untreated patients with ENL may explain the possibility of induction of excessive immune activation owning to the pre-existing high load of bacterial antigens in patients with lepromatous leprosy.

SECTION 3: MEMORY T-CELLS

The ability of inflammatory cells to respond to pathogens is crucial for maintaining healthy conditions. In mammals, lymphocytes leave the circulation and migrate to secondary lymphoid organs, such as lymph nodes, where antigens are presented. After antigen encounter, guided delivery of immune cells to sites of inflammation orchestrates host defence. Adhesion molecules control both constitutive and inflammatory leukocyte trafficking. The selectins, especially L-selectin, play a pivotal role in the initial tethering of leukocytes to the endothelium and to other leukocytes. L selectin directs lymphocytes and neutrophils to sites of inflammation. Upon T-cell activation L-selectin is shed from the leukocyte surface (Sallusto et al., 2004, Raffler et al., 2005).

In this study, the status of T-cell activation and the different subtypes of memory T-cells were investigated. In leprosy, the different classes of memory T- cells have not been studied. To my knowledge, it is for the first time that the different subtypes of memory T-cells and T-cell activation are phenotypically described in patients with ENL and LL. We are the first to study the status of T-cell activation in patients with ENL. Not only T-cell activation and the different classes of memory T-cells are described but also the changes of these immune cells over time before, during and after prednisolone treatment were investigated. Therefore, our present data will provide basic information for future studies involving T-cell activation and memory T-cells in ENL.

Activated T-cells (ENL versus LL): In our study, patients with ENL had significantly higher $CD3^+$, $CD4^+$ and $CD8^+$ activated T-cells than LL controls before treatment. However, after prednisolone treatment, T-cell activation was not significantly different in patients with ENL and LL controls except for the transient activation of $CD8^+$ T-cells. This result evidences of T-cell activation in patients with ENL reactions. *In vitro* stimulation of PBMCs from patients with ENL reaction with *M. leprae* whole-cell sonicate has shown an increased T-cell response as assessed by IFN-γ and TNF-α production. Excessive T-cell activation as a cause of tissue damage in several inflammatory diseases have been described in many studies (Baniyash, 2004, Park and Kupper, 2015). The result described here evidence of T-cell activation

in patients with ENL, implying the involvement of T-cell activation in the pathogenesis of ENL.

Activated T-cells in patients with ENL before and after treatment: In this study changes in activated T-cells was also investigated before, during and after prednisolone treatment of patients with ENL. The expression of $CD3^+$, $CD4^+$ and $CD8^+$ activated T-cells were significantly reduced during and after prednisolone treatment. The reduction of activated T-cells following corticosteroid treatment may be explained by the immuno-suppressive activity of prednisolone. Although studies showing the effect of prednisolone treatment on T-cell response in leprosy reactions is lacking, several studies in other inflammatory diseases have shown the suppressive effect of prednisolone on the T-cell response (Hepburn and Slade, 1987, Shevach, 2009, Kasang et al., 2016). Consequently, our present findings provide evidence that the effect of prednisolone treatment of patients with ENL could be through supressing T-cell responses.

Effector memory T-cells (ENL versus LL): The median percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells expressing effector memory T-cells in PBMCs of patients with ENL were significantly high compared to LL controls before treatment. Such a difference was not observed after prednisolone treatment of patients with ENL. This implies that in patients with ENL, there is a continuous activation of T-cells. This continuous T-cell activation could lead to an excess antibody-antigen complex formation but insufficient to clear bacilli from lesions (Cooper et al., 1989). This means the rate of immune-complex formation is greater than the rate of immune-complex clearance which leads to further tissue damage through recruitment of inflammatory molecules to the site of deposition. In lepromatous leprosy, there is high load of bacilli. The macrophages are laden with this intact bacilli but unable to process and present to T-cells for further action. LL patients are also characterized by the presence of high antibody titre although these antibodies play little or no role to protect the multiplication of *M. leprae* in these patients. As described in the preceding sections, spontaneous activation of T-cells could lead to the macrophage activation or B-cell activation or both. Macrophage activation results in the processing of the bacilli and releasing the processed bacterial components which further activate other immune cells. The activation of B-cells by T-cells could produce functional antibodies which

form immune-complex with the already accumulated bacterial antigens. However this scenario is less likely as it is confirmed in this study that the different subtypes of B-cells did not significantly different in ENL and LL patients. Whichever the activation takes place, if excess immune-complex is formed due to the presence of excess antigens in the body, it leads to more immune-complex formation than immune-complex clearance and hence, some immune-complexes deposit in tissues and often induce an inflammatory responses, and can cause tissue damage. The causes of tissue damage could be due to the action of complement cleavages which induces the release of tissue damaging granules such as histamine or the recruitment of inflammatory cells such as neutrophils and macrophages into the tissue. However, this assumption need further study to give definitive evidence. Previous studies have suggested that human effector memory T-cells (T_{EM}) display characteristic sets of chemokine receptors and adhesion molecules that are required for homing to inflamed tissues and they have an immediate effector function (Kaech et al., 2002, Sallusto et al., 2004). This situation could amplify the immune response and hence further aggravate tissue damage in a vicious circle.

Effector memory T-cells in patients with ENL before and after treatment: A kinetic analysis of the expression of effector memory T-cells in the PBMCs from patients with ENL before, during and after prednisolone treatment showed that the median percentage of effector memory T-cells was decreased from 27% before treatment to 8% after treatment with an effect size of 20%. It has been described in previous sections that effector memory T-cells rapidly differentiate to effector T-cells upon antigenic stimulation. The consequence of excessive expression of effector memory T-cells is detrimental to tissue damage (Farber et al., 2014). In healthy individuals, effector T cells do not express CCR7 or CD62L and do not home to lymph nodes (Flynn and Gorry, 2014). A breakdown in the compartmentalization of such effector T cells is predicted to have detrimental consequences for the immune system. Hence, the fact that an increased percentage of effector memory T-cells in patients with ENL was percent before treatment suggests that effector T-cells take part in the process of tissue damage observed in these patients.

Naïve T-cells (ENL versus LL): : Interestingly, the median percentage of naïve T-cells expression in $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the PBMCs of patients with ENL

was significantly low compared to LL controls before treatment implying that T-cells from patients with ENL have more antigenic exposure than those from LL. It is important to note that patients with ENL had LL before they developed ENL during which a high percentage of naïve T-cells is expected since higher percentage of naïve T-cells were investigated in this study. Following the development of ENL, the percentage of naïve T-cells drops to below 30%. This implies that either those previously naive T-cells became responsive and able to recognise their cognate antigen or the newly produced T-lymphocytes during the onset of ENL reactions are able to recognise and respond to the existing *M. leprae* antigens unlike in lepromatous leprosy. It is an established fact that despite the high bacterial load in patients with lepromatous leprosy, the cell-mediated immune response is virtually absent (Godal et al., 1971, Godal et al., 1972). In addition to a specific loss of cell-mediated immune response against *M. leprae* in these patients, a relative impairment of the ability of lymphocytes to react *in vitro* has also been reported. Furthermore, lymph nodes from patients with lepromatous leprosy show a deficiency of these cells in those areas associated with the development of cell-mediated immune responses (Godal et al., 1971, Godal et al., 1972, Myrvang et al., 1973, Kaplan et al., 1985, Bobosha et al., 2014b). Therefore, the significantly reduced median percentage of naïve T-cells in blood from patients with ENL reaction provides an evidence of T-cell responsiveness in patients with ENL. This means that unlike in LL patients, the naïve T-cells in ENL patients are primed in recognition of the *M. leprae* antigen.

Naive T-cells in patients with ENL before and after treatment: The median percentage of naive T-cells were significantly increased after prednisolone treatment of patients with ENL. The percentage of naive T-cells in untreated patients with ENL reactions was less than 30% and was increased to nearly 50% after treatment. Previous studies have shown that prednisolone treatment increases in a dose-dependent manner the percentage of naive T-cells in experimental mice. Hence, the finding of high percentages of naïve T-cells after prednisolone treatment of patients with ENL may be explained by the fact that prednisolone resolves inflammation at least partly by increasing the percentage of naïve T-cells which concurrently reduce the percentage of activated T-cells.

SECTION 4: MEMORY B-CELLS

Memory B-cells are subtypes of B-cells that are formed within germinal centres following infection. They proliferate and differentiate into antibody producing plasma cells also called effector B-cells in response to re-infection. Memory B-cells rapidly differentiate into plasmablasts that produce class-switched antibodies which are capable of clearing the infection far more quickly than naive B-cells (Kurosaki et al., 2015). The different classes of memory B-cells have been studied in various chronic viral infections such as hepatitis and HIV and several autoimmune diseases (Moir and Fauci, 2009, Oliviero et al., 2011). The role of B-cells in the pathogenesis of ENL has been speculated in several studies but has never been studied. For the first time, we studied B-cells and the memory B-cell sub-types in patients with ENL and LL controls at different time points (before, during and after treatment) to investigate the dynamics of these cells during the course of prednisolone treatment.

Total B-cells (ENL versus LL): The percentage of total B-cells was not significantly different in the two groups before treatment. However, after treatment, the proportion of B-cells was significantly reduced from 9.5% to 5.7% in patients with ENL. The reduction of B-cells after prednisolone treatment of patients with ENL could be either transitory or associated with the subsiding of the ENL reaction which needs further investigation. The success of Rituximab to deplete B-cells for the treatment of rheumatoid arthritis has stimulated investigation of its effects in several other immune disorders, and considerable interest in the potential of drugs that can modulate B-cell function for the treatment of such diseases (Edwards et al., 2004). Thus, the finding of reduced B-cells after ENL subsides poses the question whether depleting B-cells could be effective in treatment of ENL. Therefore, investigating the role of B-cells in the pathogenesis of ENL is needed in the future studies of ENL.

Naive B-cells (ENL vs LL): Patients with ENL had a significantly lower naïve B-cells 97.6.0%) than LL controls (84.6%) before treatment ($P \leq 0.05$; L-H=6.75). The percentage of naïve B-cells was not significantly different in the two groups after treatment. This shows that patients with LL not only have high a frequency of naïve T-cells but also have a higher frequency of naïve B-cells than patients with ENL.

Activated and resting memory B-cells (ENL versus LL): A significance difference was not observed with regard to the frequency of resting memory B cells (RM) in the two groups before treatment. However, the median percentage of activated memory B-cells (AM) was significantly higher in patients with ENL (2.1%) than in LL controls (1.4%) before treatment. Several studies have shown that activated memory B-cells are increased in patients with disease flares in systemic lupus erythematosus (SLE) (Jacobi et al., 2008) and rheumatoid Arthritis (Adlowitz et al., 2015). However, the biology of ENL and autoimmune diseases is different and whether activated memory B-cells are undesirable or not in the pathogenesis of ENL should be further investigated to arrive at a conclusive evidence.

Tissue-like memory B-cells (ENL versus LL): Patients with ENL had higher percentage of tissue-like memory (TLM) B-cells (9.3%) than LL controls (4.8%) before treatment. Thus, our finding implies that TLM could have a role in the pathogenesis of ENL and this is a new exciting area for further investigation. A similar increase in the proportion of tissue-like memory B-cells has been reported in other chronic infections such as in hepatitis C virus and malaria infections, and in certain autoimmune diseases (Weiss et al., 2009, Charles et al., 2011). Several studies have indicated that TLM B-cells represent the exhausted state of B-cells since they express several inhibitory receptors, including the immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing inhibitory receptor Fc receptor-like protein 4 (FcRL4) (Barber et al., 2006). TLM B-cells show a reduced tendency to proliferate in response to cognate antigen (Moir et al., 2010). The expression of FcRL4 on human B-cell lines disrupts immune synapse formation and blocks antigen induced B-cell receptor (BCR) signalling (Amu et al., 2007). They also express not only FcRL4 but also a number of other inhibitory and chemokine receptors that would reduce the likelihood of B and T cell interaction according to Moir et al. (2008). Kardava *et al*. have shown that a specific siRNA knockdown of FcRL4 and other inhibitory receptors may lead to a rescue of Ig secretion and proliferation in these tissue-like memory B-cells (Kardava et al., 2011). Since the identification of an exhausted population of B-cells (TLM) phenotypes have been witnessed in other chronic diseases such as malaria and hepatitis-C (Weiss et al., 2009, Charles et al., 2011), it appears that the higher frequency of TLM B-cells in ENL could alter the B-cell/T-cell interaction in these patients through blocking B-cell receptors and this hypothesis could be a fertile area

for future investigation. Once, this hypothesis is proved, the search for the B-cell immunomodulators that safely overcome this exhaustion phenotype may be necessary in order to develop proper immune response to this complicated disease.

SECTION 5: *IN VITRO* CYTOKINES PRODUCTION AND GENE EXPRESSION

To investigate the *in vitro* cytokine response to *M.leprae*, PBMCs from patients with ENL and LL controls were cultured with *M.leprae* whole-cell sonicates (MLWCS), Phytohaemagglutinin (PHA) or no stimulation for 6 days. The cytokine levels in culture supernatants were determined using sandwich ELISA specific to TNF-α, IFN-γ, IL-β, IL-6, IL-8, IL-17A and IL-10. The gene expression of these cytokines in whole blood and skin biopsies from patients with ENL and LL controls before and after treatment was also investigated.

The *in vitro* cytokine response of PBMCs from patients with ENL and LL controls to *M. leprae* antigen and its gene expression in blood and skin biopsy samples were compared before, during and after treatment using a t-test. Some of the key findings are described in the following paragraphs.

TNF-α : TNF-α is a cell signalling cytokine involved in systemic inflammation and it is one of cytokines that contribute to the acute phase reaction. However, excessive production of TNF-α can cause tissue injury. The *in vitro* response of TNF-α to *M.leprae* whole cell sonicate (MLWCS) in the PBMCs from patients with ENL and LL controls was investigated before and after treatment. The mean production of TNF-α in response to *M. leprae* antigen was significantly higher (83.6.4 ±18.8, SE pg/mL) in the culture supernatants of PBMCs from patients with ENL than from LL patient controls (19.4pg/mL ±10.44) before treatment. However, TNF-α production was not significantly different in the groups after treatment. Similar findings have been reported by several studies (Barnes et al., 1992a, Santos et al., 1993, Sampaio et al., 1998b). On the other hand, the detection of TNF-α in plasma or serum samples from patients with ENL has not been consistently reported. Some authors did not find a significant difference in patients with ENL and LL controls (Lockwood et al., 2011, Madan et al., 2011a). Others reported that increased TNF-α production occurred during corticosteroid treatment and decreased after treatment (Moubasher et al., 1998a,

Haslett et al., 2005, Villahermosa et al., 2005, Stefani, 2009). Interestingly, an upregulation of TNF-α production after thalidomide treatment of patients with ENL was also reported by Haslett et al. (2005). These authors justified the increasing production of TNF-α during thalidomide treatment as an indication of immune stimulation. However, thalidomide and prednisolone may have different effect on the TNF-α. The variation of the results in various studies can be attributed to several factors such as experimental design, sample size, ENL definition, assay sensitivity and assay methods.

In our study, the mRNA gene expression for TNF-α in blood and skin biopsies from patients with ENL was significantly upregulated compared to in LL controls before treatment. After treatment, the level of gene expression for TNF-α in blood and skin biopsy samples of patients with ENL was significantly decreased. Similar findings have been reported by previous studies (Barnes et al., 1992b, Sampaio et al., 1998b, Moraes et al., 1999b, Teles et al., 2002). On the other hand, the absence of any significant difference regarding the gene expression of TNF-α in the skin biopsies from patients with ENL and LL controls was also reported by Yamamura et al. (1992).

Thus, our present kinetic studies confirmed that TNF-α is certainly increased during ENL reactions in the same individual both *in vitro* (following stimulation of the cells with *M.leprae* whole cell sonicate) and *in vivo* (increased TNF-α gene expression *in situ*). Hence, our data imply that TNF-α is involved in the pathogenesis of ENL and it may be of use for the diagnosis of ENL. Investigating the sources of TNF-α (identifying the major immune cells producing TNF-α) in the pathogenies of ENL may also be important to explore alternative therapeutics in the future.

IFN-γ: IFN-γ is a major macrophage activator and a known inducer of macrophage TNF-α. It increases antigen presentation and lysosome activity of macrophages. However, over expression of INF-γ has been associated with the pathogenesis of a number of inflammatory and autoimmune diseases (Lee et al., 2012).

In the current study, the mean production of IFN-γ in response to *M. leprae* antigen was significantly higher (1361pg/mL ± 309.6) in the culture supernatants of PBMCs from patients with ENL than from LL patient controls (280.1pg/mL ± 309.6) before treatment ($P \leq 0.05$). However, IFN-γ production was not significantly different in the

groups after treatment of patients with ENL. Previous independent studies have also reported an increasing *in vitro* production of IFN-γ in untreated patients with ENL to the response of *M.leprae* stimulation (Cooper et al., 1989, Sreenivasan et al., 1998, Iyer et al., 2007). IFN-γ mRNA gene expression in the blood and skin biopsies from patients with ENL was significantly upregulated compared to in LL controls before prednisolone treatment. After treatment, the level of IFN-γ gene expression in the blood and skin biopsy samples from patients with ENL was significantly decreased. Similar findings have been reported by Moraes et al. (2000) Iyer et al. (2007).

It should be noted that although fewer investigations have focused thus far on the role of IFN-γ, in contrast to the weight given to TNF-α, the results are more consistent and indicate an important role of IFN-γ in the immunopathology and occurrence of ENL. It worth to mention that administration of recombinant IFN-γ to patients with lepromatous leprosy led to the development of ENL reactions in 60% of the patients over a 6-7 months period compared with an incidence of 15% per year with multiple drug therapy alone (Sampaio et al., 1992). Previous studies have demonstrated that monocyte/macrophage TNF-*α* production can be enhanced by the synergistic effect of IFN-γ (Billiau, 1996). In addition, IFN-*γ* priming of peripheral blood monocytes from patients with leprosy has been demonstrated to enhance TNF-*α* production, both *in vivo* and *in vitro* (Sampaio et al., 1992). The mechanism by which IFN-γ induces ENL reactions in patients with leprosy is suggested to be through priming of monocytes, resulting in enhanced TNF-α production (Sampaio and Sarno, 1998).

IL-6: In the present study, we found that the *in vitro* response of IL-6 production was substantially higher in patients with ENL than in LL controls before treatment. IL-6 mRNA gene expression in the blood and skin biopsy samples was also significantly upregulated in patients with ENL than in LL controls. The *in vitro* production and gene expression of this cytokine was decreased after prednisolone treatment of patients with ENL and a significant difference was not observed in the two groups after treatment. However, its gene expression in the skin samples was not appreciably decreased in patients with ENL after prednisolone treatment. This suggests that although the systemic symptoms of ENL subside after treatment, there could be an ongoing immune response locally in the skin lesions which could take a longer time to establish immune-homeostasis. Similar findings have been reported by Yamamura et al. (1992)

and (Moraes et al. (1999b), 2000, 2001). IL-6 is an interleukin that acts as both a pro-inflammatory and an anti-inflammatory cytokine which is secreted by T cells and macrophages (Scheller et al., 2011). It is an important cytokine mediating fever and the acute phase response through its ability of crossing the blood-brain barrier and initiating the synthesis of prostaglandin E2 (PGE2) in the hypothalamus thereby changing the body's temperature set point (Banks et al., 1994). Hence, it is sensible to assume that higher levels of IL-6 production could contribute to the development of ENL reactions in non-reactional lepromatous leprosy patients mainly owing to the potent pro-inflammatory role of IL-6 and its capacity to stimulate antibody production. The increased production of IL-6 in ENL reactions due to polymorphisms in the genes encoding the cytokine have been suggested (Sousa et al., 2012). Based on the multiple effects of IL-6 on the control of innate and adaptive immune responses, its potential contribution to the immunopathogenesis of ENL reaction needs to be explored further.

IL-8: IL-8 (CXCL8) is a chemokine produced mainly by macrophages and plays a key role in inflammation in neutrophil recruitment and inducer of phagocytosis. Studies have shown that anti-IL-8 treatment prevent neutrophil-dependent tissue damage as well as neutrophil infiltration in several types of acute inflammatory reactions, including lipopolysaccharide (LPS)-induced dermatitis, LPS/IL-1-induced arthritis and acute immune complex-type glomerulonephritis (Harada et al., 1994).

In this study, higher *in vitro* production of IL-8 to the response of PBMCs stimulation to *M.leprae* was obtained in patients with ENL than in LL controls before, during and after prednisolone treatment. Unlike the other cytokines studied, IL-8 production did not decrease during and after prednisolone treatment of patients with ENL. The mRNA gene expression of IL-8 both in the blood and skin biopsy samples was also significantly upregulated in patients with ENL before, during and after prednisolone treatment. A Few studies have investigated the role for serum IL-8 in the immunopathogenesis of ENL by comparing its production and gene expression. IL-8 mRNA upregulation in untreated patients with ENL has been reported by few studies (Yamamura et al., 1992) and *in vitro* production (Goulart et al., 2000) and both studies are in agreement with our finding.

Thus, the finding of increased IL-8 production as well as IL-8 mRNA gene expression in the blood and skin lesions from patients with ENL suggest a potential role for this chemokine in the pathogenesis of ENL. Hence, exploring the potential role of IL-8 in the immunopathogenesis of ENL may provide a valuable information in the diagnosis and treatment of ENL.

IL-17: IL-17A is an immunoregulatory cytokine capable of promoting the generation of pro-inflammatory cytokines and chemokines, which leads to the attraction of neutrophils and macrophages to the inflammation site (Jin and Dong, 2013). The *in vitro* production of IL-17A in PBMC samples and IL-17 mRNA gene expression in the blood and skin biopsy samples from patients with ENL was significantly increased before treatment and declined after prednisolone treatment. The upregulation of IL-17A before and after thalidomide treatment of ENL was reported by Martiniuk et al. (2012). The number of patients involved and the assay controls (such as the housekeeping genes and patient controls) used in that study contribute to the observed differences with our results. Martiniuk *et al*. performed a gene expression study in 7 ENL biopsies pre- and post-treatment with thalidomide however, not only was a larger group of 46 patients with ENL used here but also IL-17A mRNA gene expression was studied in blood and skin biopsy samples before and after prednisolone treatment. More importantly, in the Martiniuk *et al*. study, controls were not included and also thalidomide and prednisolone may have different effect on IL-17 production.

The finding of increased IL-17A production and its mRNA gene expression in patients with ENL in the present study shows the involvement of IL-17A in the pathogenesis of ENL reaction. Hence, understanding the exact role of this cytokine in ENL reaction will benefit the development of novel immune modulators that reduce the inflammatory tissue damage occurring in patients with ENL.

IL-10: IL-10 is a well-known cytokine involved in downregulating macrophage functions. IL-10 has been shown to inhibit cytokine synthesis by monocytes, namely TNF-α, IL-1, IL-6, IL-8, IL-12 (Sampaio et al., 1998b, Sampaio et al., 1998a). Thus, the finding of decreased IL-10 production as well as IL-10 gene expression in untreated patients with ENL implies the loss of control over the above mentioned pro-inflammatory cytokines which exacerbates ENL reactions.

The *in vitro* production of IL-10 in response to *M.leprae* stimulation in the PBMCs of patients with ENL was significantly lower than in LL controls before treatment. After prednisolone treatment of ENL patients, IL-10 production was significantly increased and it was higher than the value obtained in LL controls. The present result is in agreement with (Sampaio et al., 1998b). Although IL-10 mRNA gene expression in the blood samples before and after treatment in patients with ENL and LL controls did not reveal a statistically significantly different result, the longitudinal comparison in patients with ENL has shown significantly increased IL-10 mRNA gene expression after prednisolone treatment to similar to the *vitro* IL-10 production. On the other hand, unlike the blood samples, the gene expression in skin biopsy samples was significantly decreased before treatment in patients with ENL compared to LL controls and considerably increased after prednisolone treatment. Quantitative IL-10 mRNA gene expression studies were not available to compare with the results obtained in this study. Variable and inconstant reports have been published on serum or plasma IL-10 production in patients with ENL. An Indian study reported increased IL-10 production in patients with ENL than in patients with T1Rs (Moubasher et al., 1998b). However, the authors made a comparison between patients with ENL and T1Rs taking T1Rs as the controls. A similar report was made by Madan et al. (2011b). Others studies failed to detect any differences in serum IL-10 between patients with ENL and LL controls (Iyer et al., 2007a, Stefani et al., 2009, Attia et al., 2014) or in IL-10 mRNA in biopsy skin specimens (Moraes et al., 1999a). The variable and inconsistent reports on IL-10 can be accounted for by several factors such as sample size, case definitions, inclusion and exclusion criteria, assay methods, assay specificity and sensitivity and use of appropriate case controls.

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SECTION 6. CIRCULATING C1-Q AND ITS GENE EXPRESSION

The amount of circulating C1q in the plasma of patients with ENL and LL was quantified using a C1q ELISA. The gene expression of C1q was also quantified in blood and skin biopsy samples of these patients before and after treatment.

Patients with ENL had significantly lower circulating concentrations of C1q than LL controls before treatment. However, after treatment, the amount of circulating C1q was not significantly different in both groups. A similar result has been reported in an earlier study by de Azevedo and de Melo (1966). The decreased frequency of circulating C1q in the sera of patients with ENL could be due to its utilisation by the antigen-antibody complex formation. Similar decreased serum complement levels have been observed in other immune complex disorders such as acute glomerulonephritis (Lange et al., 1960, Lewis et al., 1971, Ohi and Tamano, 2001) and acute systemic lupus erythematosus (SLE) (Baatrup et al., 1984, Grevink et al., 2005). However, the pathophysiology of autoimmune disorders and infectious diseases are different. In autoimmune disorders, the finding of an antigen-antibody complex may be conclusive enough since the autoantibodies are formed as part of the pathogenesis. However, in any infectious diseases like ENL, the finding of antigen-antibody is part of the host defence mechanism against the infection. Circulating immune complexes (ICs) are produced continuously in response to infection and immune reactions to foreign antigens. In such cases most ICs are of little pathologic significance because they are rapidly cleared. This cycle of IC formation and clearance is an important component of acquired immunity, ensuring removal or processing of antigens (Schifferli and Taylor, 1989, Stokol et al., 2004, Mayadas et al., 2009). However, the failure of immune-complex clearances leads to immune-complex deposition. Immune-complex deposition initiates a complex cascade of events, which culminate in the recruitment and activation of neutrophils and macrophages which in turn produce pro-inflammatory cytokines and other inflammatory molecules causing tissue damage. Therefore, to conclude the antigen-antibody complex (immune-complex) formation as the aetiology of the ENL, one has to prove the failure of antigen-antibody clearance or clearly demonstrate immune-complex deposition in the skin lesions.

The gene expression levels of *C1q A*, *B* and *C* in the blood and skin biopsy samples from patients with ENL and LL controls were not significantly different before treatment. However, after treatment, the gene expression levels of *C1q* A, B and C were increased in the blood samples. C1qA mRNA expression was increased in the skin biopsy samples of patients with ENL compared to LL after treatment. On the other hand, The C1qC mRNA expression was decreased in skin biopsy samples of patients with ENL compared to LL patient controls after treatment. Studies on C1q gene expression in leprosy are not available to make comparisons with these results.

In this study, the increased C1q production in the plasma samples of patients with ENL after treatment could be due to increased C1q gene expression in blood samples of these patients after treatment. The lower quantity of circulating C1q in the plasma may be act as a positive feedback for C1q gene expression. It has been reported that the inflammatory cytokines such as IFN-γ and IL-6 increase C1q production by macrophages (Lu et al., 2008). Several studies indicated that C1q can modulate dendritic cell maturation, pro-inflammatory cytokine production, and T- and B-cell responses in addition to its classical function to initiate complement activation (Fraser et al., 2006, Castellano et al., 2007, Nayak et al., 2012). Recently, increased circulating C1q and C1qC gene expression in patients with active tuberculosis compared to healthy controls and individuals with latent TB infection has been reported by Cai et al. (2014) indicating its potential as a biomarker to discriminate active TB from latent TB cases . Therefore, C1q could be a potential diagnostic marker for ENL in the same way, which necessities further investigation.

SECTION 7. THE LEVELS OF ANTI PGL-1, LAM AND AG85 ANTIBODIES

The levels of anti ND-O-BSA (PGL-1), LAM and Ag85 antibodies were measured by ELISA in the plasma samples of 30 patients with ENL and 22 non-reactional LL controls before and after prednisolone treatment of ENL cases. The first sample was obtained from each patient before administering MDT in either groups. The laboratory assays were done at John Spencer's laboratory, Colorado State University, CO, USA.

PGL-1: The PGL-1 levels were not significantly different in patients with ENL (OD= 1.430 ± 0.1281) and LL controls (1.341 ± 0.1415) ($P>0.05$) before treatment. However, after treatment the level of PGL-1 was significantly decreased (OD=1.183 ± 0.1333) in patients with ENL compared to LL patient controls (OD= 2.091 ± 0.1081) ($p < 0.0001$). A study in Brazilian leprosy patients including 5 untreated ENL cases, 13 non- reactional LL patients and 13 other clinical forms of leprosy has reported that the levels of PGL-1 were not significantly different in ENL and LL patients (Silva et al 2007) which is in agreement with the present result. The finding of lower level of PGL-1 in ENL cases than in the corresponding LL controls after prednisolone treatment of ENL cases in this study could be explained by the effect of prednisolone treatment. Previous studies have shown that the level of PGL-1 was significantly decreased in ENL cases treated with prednisolone compared to LL patients (Raju et al., 2014). In a comparison made between acute and chronic ENL cases, acute ENL cases had higher levels of PGL-1 than chronic ENL cases before treatment ($P\leq0.001$). The differences in the levels of PGL-1 in acute and chronic cases could be attributed to the clinical condition of these patients and their treatment status. Higher rate of *M. leprae* multiplication is expected in untreated acute ENL cases than in chronic cases. PGL-1 is synthesised by viable and actively dividing *M. leprae* (Lobato et al., 2011, Spencer and Brennan, 2011). Therefore the finding of a higher anti-PGL-1 antibody titre in acute ENL than in chronic ENL in this study indicates the higher rate of PGL-1 synthesis in acute ENL cases than in chronic ENL cases. Similar findings has been reported by Bhoopat et al. (1991). After treatment, the levels of PGL-1 still remain lower in chronic ENL cases than in acute ENL cases this could be due to the continuation of prednisolone treatment in chronic cases as described above.

LAM: Similar to PGL-1, the levels of LAM were not significantly different in patients with ENL (OD=1.191 ± 0.09790) and LL controls (OD= 1.116 ± 0.1183) before treatment (P>0.05). However, after prednisolone treatment of ENL cases the levels of LAM were lower in ENL cases (OD= 1.095 ± 0.08101) than in LL controls (OD=1.592 ± 0.08426) (P≤0.0001). The reduction of the level of LAM in ENL cases could be due to the effect of prednisolone treatment as previously reported by Raju et al (2104).

Ag85 (ML2028): Antigen 85 (Ag85) complex proteins are major secretory products of *Mycobacterium.* Like LAM, similar level of Ag85 was measured in the plasma samples from patients with ENL (OD=1.154 ± 0.1218) and LL controls (OD =1.134 ± 0.1360). After prednisolone treatment of ENL cases the level of Ag85was lower in ENL cases (OD= 0.9713 ± 0.1164) than in LL controls (OD= 1.536 ± 0.1108) (P≤0.001). The differences in the levels of Ag85 after treatment in the two groups might be due the effect of prednisolone treatment of ENL cases. However, this assumption should be further investigated and supported by definitive evidence.

In this study, we did not included healthy endemic controls and hence it was not possible to extrapolate the results obtained to the general population. The purpose of the study was to compare the levels of anti ND-O-BSA (PGL-1), LAM and Ag85 antibodies obtained from patients with ENL with that of non-reactional LL controls. The assumption was at least one of these antibodies could be used to predict who is going to develop ENL among those non-reactional LL patients. Nevertheless, the exclusion of endemic health controls can be considered as one of the limitations of the study.

What drives ENL? A stepwise illustration has been give below to show what drives ENL (Figure 7.1A-D)

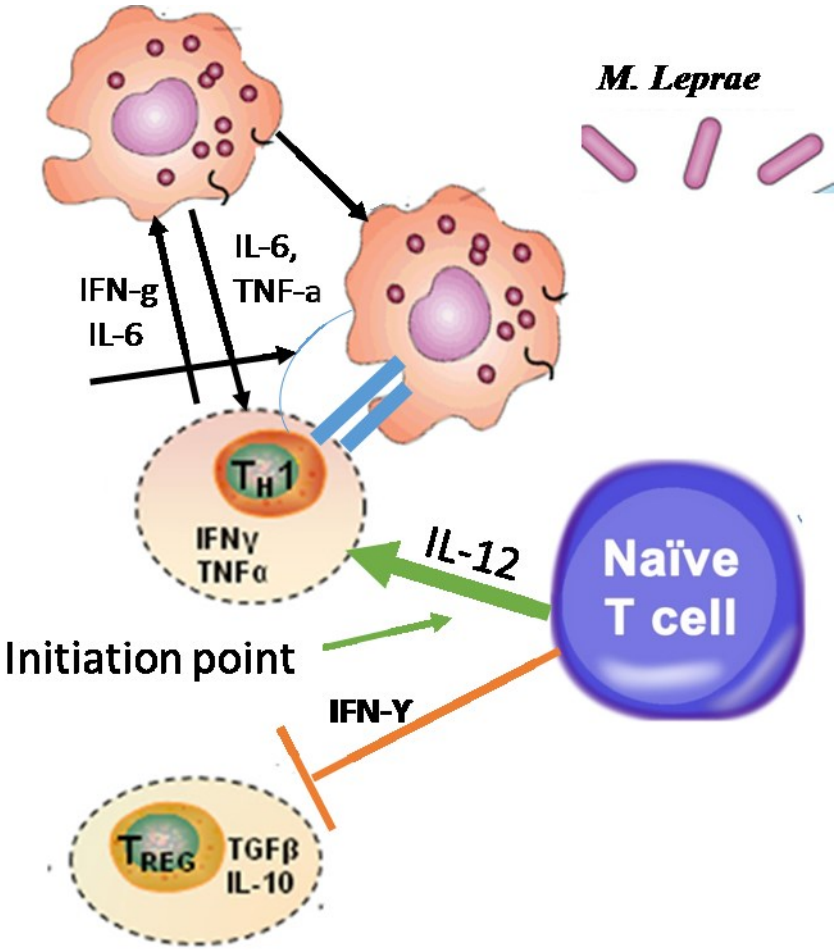

Figure 7.1A: Naïve T-cells activated and polarized to Th1 through IL-12 production by INF-Ɣ dependent Tregs downregulation. Then, Th1 T-cells produce INF-Ɣ and IL-6 to activate the macrophages which are already Landen by *M.leprae*. Activated macrophages in turn present *M.leprae* to Th1 cells for further process.

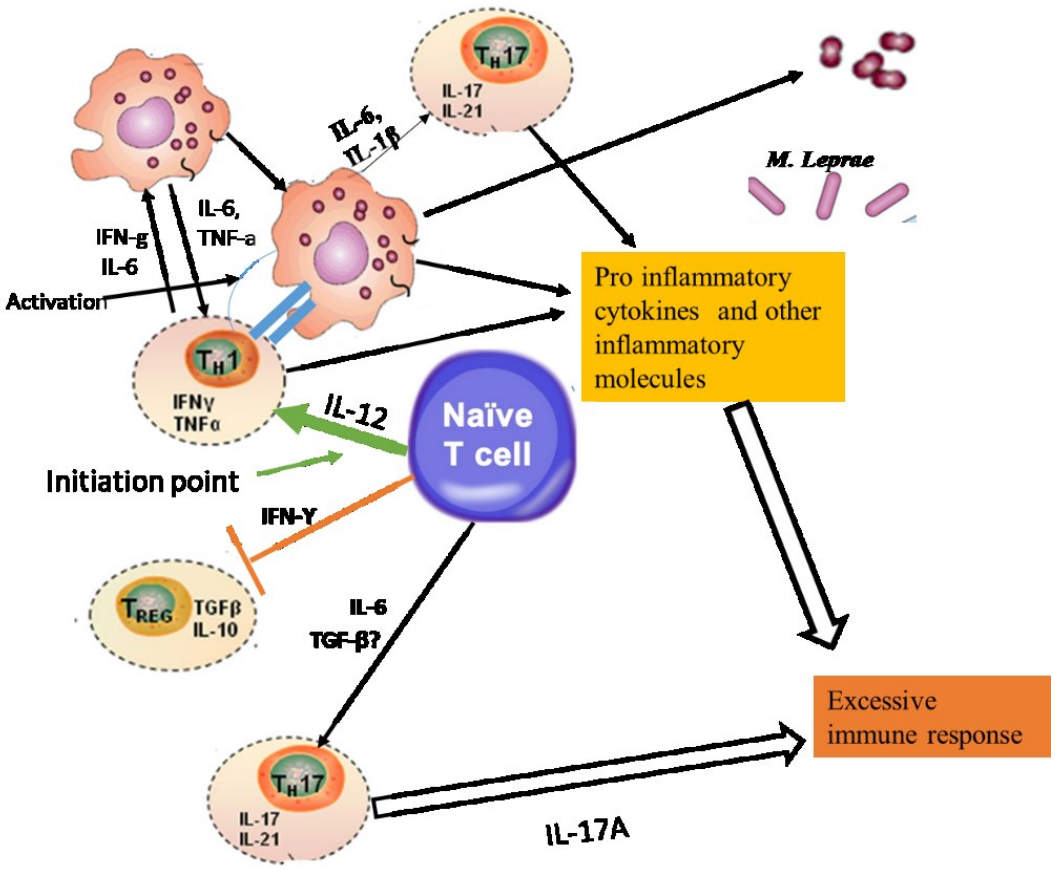

Figure 7.1B. The activation of macrophages and naïve T-cells lead to the activation of Th-17 through IL-6 and IL-1β production. Pro-inflammatory cytokines and other inflammatory molecules released to the environment by Th1, macrophages and Th17 produce excessive immune response. Furthermore, macrophages process *M.leprae* and release components of *M.leprae* to the external environment which could trigger antigen-antibody formation.

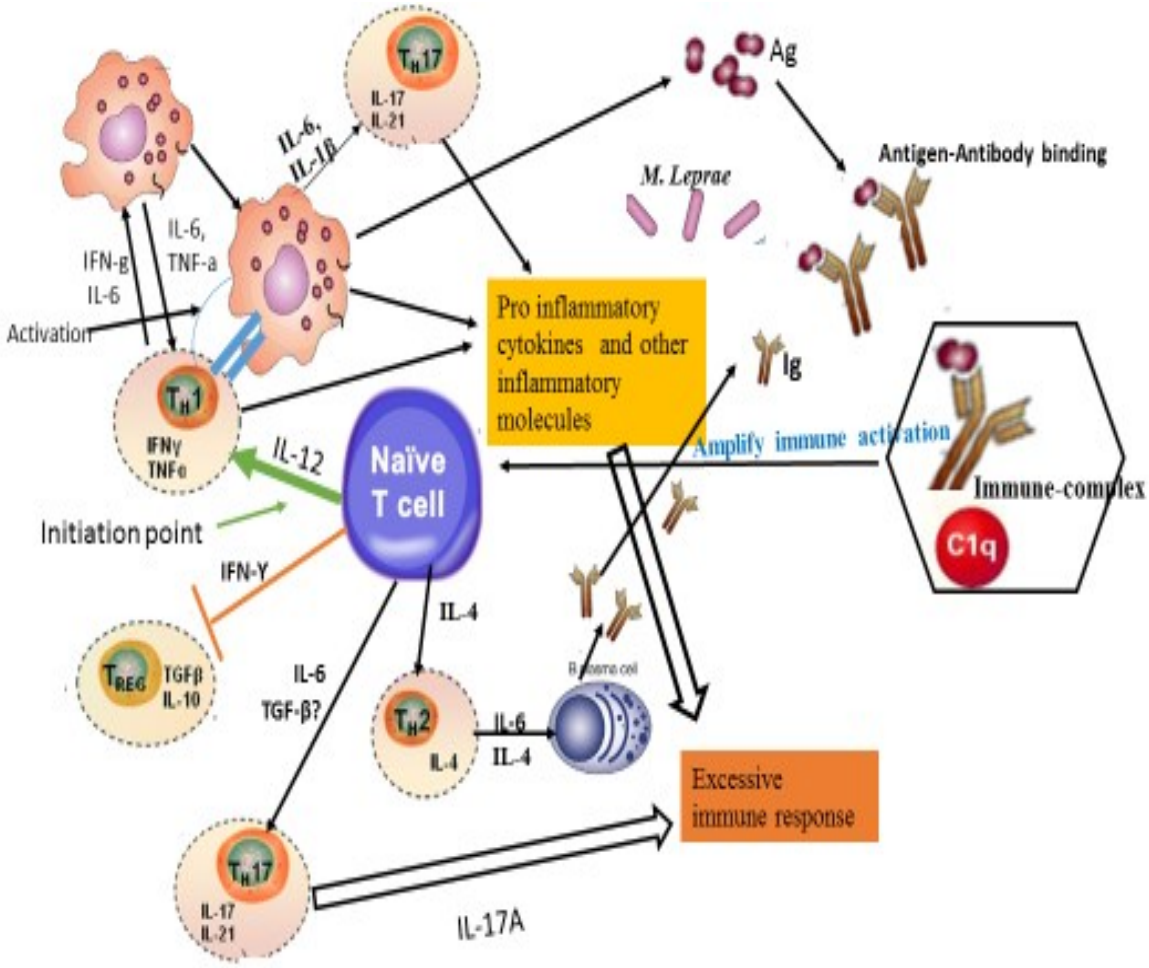

Figure 7.1C: *M.leprae* components released from the macrophages form antigen-antibody binding. In patients with lepromatous leprosy (LL) excess antibodies are produced although these antibodies do not control the multiplication of *M.leprae* in the host cells. The formation of antigen-antibody binding recruits complement C1-q and results in antigen-antibody complex formation which in turn amplify the immune activation through IL-4 production by naïve T-cells to activate Th2 cells. Then, Th2 cells initiate the production of plasma B-cells through IL-4 and IL-6 cytokines. Plasma B-cells trigger continues production of immunoglobulins (Ig) and these immunoglobulins are used for the antigen biding which are released from the macrophages.

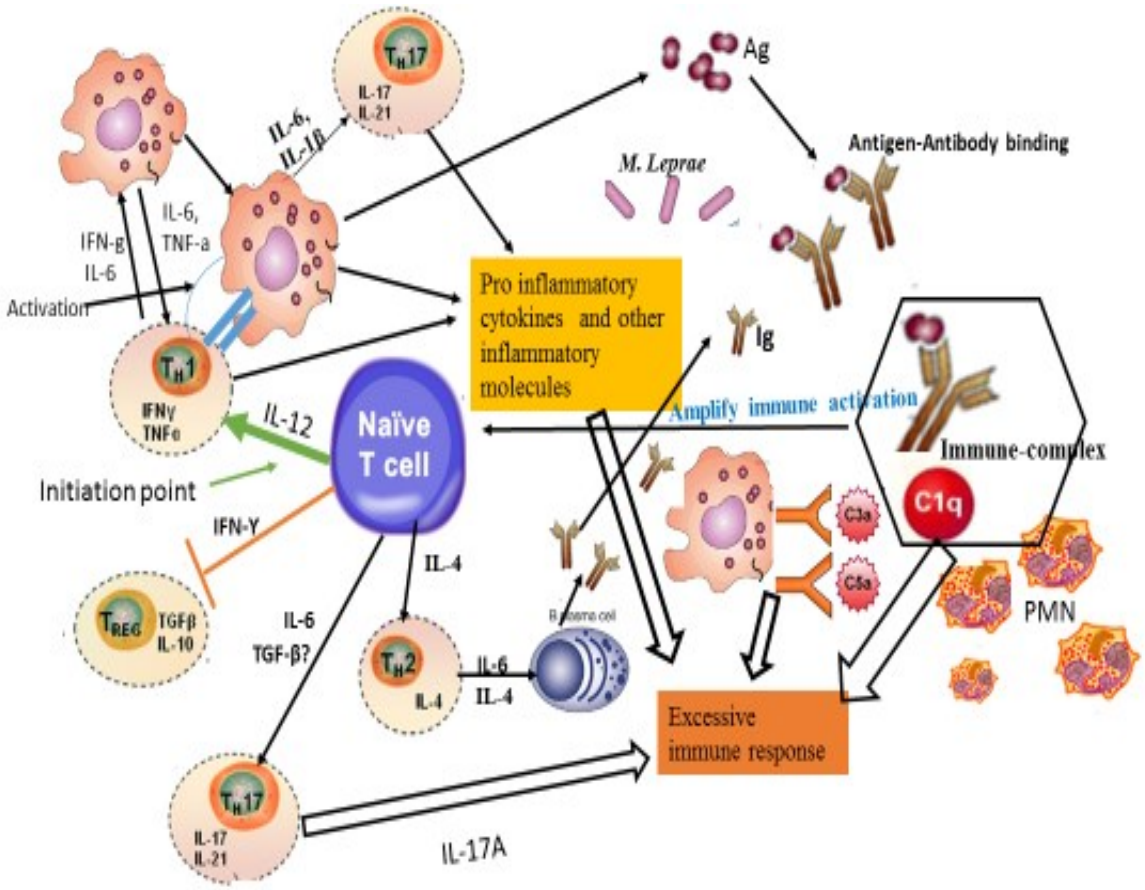

Figure 7.1D: The formation of antigen-antibody complex recruits neutrophils resulting in immune-complex formation which leads to excessive immune response.

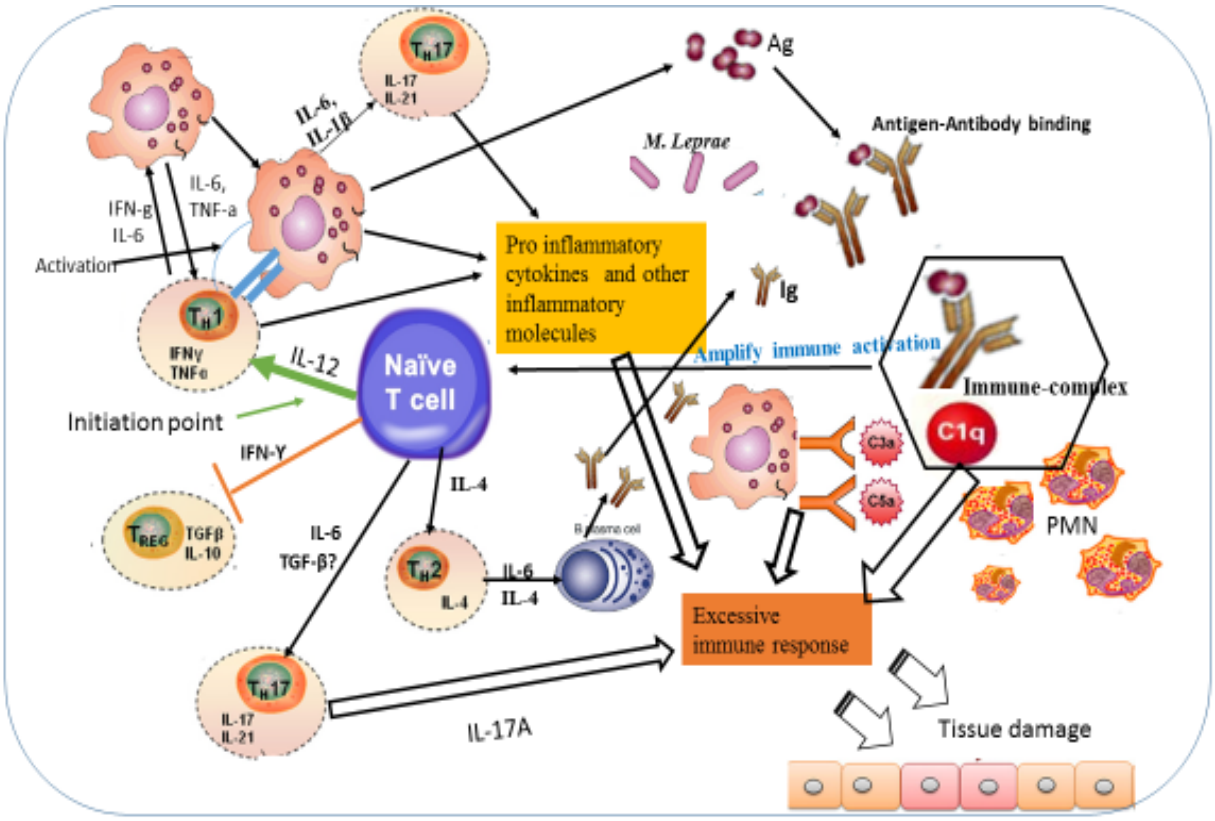

Figure 7.1E. Illustration of the possible immunopathogenesis of ENL based on the present findings (Figure 71A-D combibed).

Based on the finding of this study, the immunepthaogenesis of ENL can be illustrated using the following key steps:

1. Spontaneous activation of T-cells leading to activation of macrophages which are already loaded with intact *M. leprae*. Then, the activation of macrophages produce three key events: processing *M. leprae* and releasing of the processed antigens; antigen presentation to Th1 and production of pro-inflammatory cytokines such as IFN-Y and TNF-α and other inflammatory molecules. The causes of spontaneous T-cell activation should be investigated in the future.
2. Antigen presentation to Th1 stimulates Th1 to produce chemokines which recruits macrophages to the site of antigen deposition, pro-inflammatory cytokines (IFN-Y, TNF-α) and other inflammatory mediators which increase expression of vascular adhesion molecules.

3. The processed and released antigens are bind to the pre-synthesized antibody leading to antigen-antibody complex formation which in turn recruits C1q complement and hence immune-complex formation. Following the immune-complex formation, neutrophils will be recruited to the site of immune-complexes.
4. Once immune-complex is formed, it amplifies the immune response which leads to aggressive antigen presentation, immunoglobulin synthesis and activation of other inflammatory T-cells.
5. The pro-inflammatory cytokines and other inflammatory molecules released from macrophages, Th17 and Th1 and the immune-complex formation beyond clearance lead to tissue damage as sketched above (Figure 7.1).

CHAPTER 8: CONCLUSIONS AND RECOMMENDATION

8.1. CONCLUSION

Our understanding of the causes of ENL remains poor. The long-standing hypothesis of immune complexes as the causes of ENL has not been convincingly supported with good evidence. The contribution of cell mediated immunity in the pathogenesis of the diseases has been suggested but not yet supported by good evidence. Thus, we have hypothesized that ENL reaction is driven by the activation of previously anergic T-cells following the decreasing frequency of regulatory T-cells.

By immunophenotyping T-cell subsets by flow cytometry, we found that a significant reduction of the percentage of $CD4^+$ regulatory T-cells and an increased percentage of $CD4^+/CD8^+$ T-cells ratio in untreated patients with ENL compared to the LL controls. These findings suggest that ENL is associated with a reduced percentage of regulatory T-cells and increased $CD4^+/CD8^+$ T-cell ratio. This immune imbalance could lead to the initiation of ENL reactions either by permitting increased production of antibodies critical to immune-complex formation or as a cell-mediated immune response in patients with leprosy.

The frequency of activated T-cells and memory T-cell subtypes were also assessed to see if the reduction of the percentage of regulatory T-cells is associated with T-cell activation in patients with ENL. The percentage of activated T-cells, activated and central memory T-cells was significantly increased in patients with ENL before prednisolone treatment. This is the first study to show T-cell activation and the different subsets of memory T-cells in patients with ENL. This study illuminates the role of T-cell activation in the pathogenesis of ENL reaction and challenges the dogma of immune-complexes as the sole aetiology of ENL reactions.

To further investigate the kinetics of pro-inflammatory cytokines, the *in vitro* cytokine production of PBMCs to the response of *M.leprae* whole cell sonicate stimulation and the gene expression level in blood and skin biopsies were determined in these patients. The *in vitro* production of the cytokines: TNF-α, IFN-γ, IL-1β and IL-17A were significantly increased in untreated patients with ENL at recruitment. However, IL-10 production was significantly lower in untreated patients with ENL and significantly increased after treatment. The mRNA expression in blood and skin biopsy samples of

TNF-α, IFN-γ, IL-1β, IL-6 and IL-17A significantly reduced in patients with ENL after treatment, while mRNA expression for IL-10 was significantly increased both in blood and skin biopsy samples after treatment. This is the first study examining the effect of prednisolone on the kinetics of inflammatory cytokines in patients with ENL reactions before and after treatment. Our findings suggest that prednisolone modulates the pro-inflammatory cytokines studied here either directly or through suppressing the immune cells producing these inflammatory cytokines. This needs further confirmation through identification of the immune cells producing these cytokines.

8.2. RECOMMENDATIONS

Based on the current findings, the following recommendations for future research are made:

Regulatory T-cells: Investigating the *in vitro* suppressive function of human Tregs in untreated patients with ENL. In this study, the frequency of Tregs was described. In addition to the reduction in percentage, it is also important to know if these Tregs are functionally defective or not to draw a conclusive conclusion. Looking into the genetic polymorphism of FOXP3 in patients with ENL is also another fertile area which needs future investigation. Previous evidence has shown that the FOXP3 genetic polymorphism is involved in the pathogenesis of several diseases. Therefore, the association between single nucleotide polymorphisms (SNPs) in the FOXP3 gene and susceptibility to ENL should be investigated.

Pro-inflammatory cytokines: Delineating the cell sources of pro-inflammatory cytokines which have been identified as associated with ENL reaction. Identifying the sources of pro-inflammatory cytokines in patients with ENL may be useful for designing future studies aiming to block or modulate immune cells producing pro-inflammatory cytokines during ENL reaction.

Immune-complex: Immune-complex formation and deposition in the skin lesions of ENL should be investigated by immunohistochemistry. The formation of immune-complex in infectious disease like ENL does not account or its harmful effect since immune-complex formation is part of the host immune response to infection. Showing defects in immune-complex clearance would be important evidence. Hence, future studies should measure the rate of immune-complex clearance.

Longitudinal study: Longitudinal cohort studies are required to get a complete picture of neutrophils in the pathogenesis of ENL since the involvement of neutrophils in the pathogenesis of ENL are thought to be affected by the timing of sampling of patient materials. To investigate the effect of the timing of sampling on neutrophils population in ENL patients, the time at which the first ENL reaction

occurs should be clearly known. Then samples should be taken within a defined time interval to see the changes in neutrophils population over time in one episode of ENL. This need a well-designed longitudinal cohort study consisting of lepromatous leprosy patients and following the cohort until any of the individuals develop ENL reaction. When some individuals develop ENL, samples should be collected within the pre-set time interval for investigation.

Cytokine gene expression: Investigating the gene expression-signatures of candidate markers such as FOXP3, Neutrophils, IL-6, TNF-α and IFN-γ in lepromatous leprosy patients with and without reactions. This includes the search for a prediction tool to identify who is going to develop ENL among lepromatous leprosy patients.

Inclusion of health endemic controls: Including healthy endemic controls may be useful to obtain additional data and to further extrapolate the results to the general population. Hence, it is worthwhile to include health endemic controls in future studies.

8.3. THE WAY FORWARD

Circulating T cells in ENL show several irregularities. T-cells are disturbed in homeostasis and skewed toward activated memory and pro-inflammatory T cell types with marked reduction of Tregs. This knowledge might allow specific and targeted therapies in the future. At present, corticosteroid and thalidomide (in some countries) are the only available treatments and patients always develop several side effects. Based on our current understanding of the disease, theoretically one could think of alternative treatment strategies such as cell-based therapy with *ex vivo* expansion of Tregs and subsequent infusion of these cells could potentially restore tolerance and T-cell homeostasis in patients with ENL. However, recent findings have indicated that human Tregs can convert to pro-inflammatory IL-17 producing T-cells in the presence of specific cytokines such as IL-1β, IL-21, IL-23 (Koenen et al., 2008). Hence, administration of Tregs to these patients may enhance the diseases rather than restoring T-cell homeostasis due to the nature of Treg plasticity.

Therefore, control of excessive T-cell activation might pose an attractive therapeutic option. This could be achieved through blockage of costimulatory pathways such as

CD28/CD80. Blockage of CD28/CD80 has successfully been used in rheumatoid arthritis (Genovese et al., 2005). However, we have little information on the status of the expression of these costimulatory molecules in ENL. In addition, the presence of $CD4^{+}CD28^{-}$ T-cell population should be investigated in patients with ENL since these cells found to secrete pro-inflammatory cytokines independent of the CD28/CD80 pathway.

It might be also important to think of to interfere with T-cell trafficking into tissues and thereby reducing inflammation in these patients. Studies have shown that patients with inflammatory bowel disease and multiple sclerosis were successfully treated by administering Natalizumab, a humanized recombinant monoclonal antibody (Stuve et al., 2008). Natalizumab targets α4-integrin, which has a role in the adhesion of leukocytes to vascular endothelial cells, and thus inhibits leukocyte migration (Stuve et al., 2008). Hence, the basic mechanism of blocking leukocyte adhesion might be an attractive approach in patients with ENL to reduce inflammation and tissue destruction in the future.

In our present work, we have shown that ENL reaction is associated with reduction of Tregs and increased T-cell activation. Our findings suggest that ENL reaction is a T-cell mediated pathology. Hence, the T-cell-mediated pathology of ENL will provide further insights into disease mechanisms and will potentially result in promising new therapeutic options. Therefore, future ENL studies should consider these fertile areas to improve the treatment and management of ENL.

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APPENDICES

Appendix 1: Ethical approvals, Support letter and Material Transfer Agreement

- AHRI/ALERT Ethical Review Committee Approval sheet
- LSHTM Observational Research Ethics Committee Approval letter
- The National Research Ethics Review Committee approval (Ethiopia)
- Support letter from ALERT Hospital
- Material Transfer Agreement Memorandum

AHRI-ALERT ETHICAL REVIEW COMMITTEE APPROVAL SHEET

TITLE OF THE PROJECT

"The immunopathology of Erythema Nodusum Leprosum (Impa ENL)."

PI: Edessa Negera

Project Reg. No. PO32/12

Recommendation of the AHRI-ALERT Ethics Review Committee

The request for an initial review on the above mentioned research project was duly considered and approved by the AHRI/ALERT Ethics Review Committee on its meeting on November 06, 2012. The PI should submit Progress report of the work every 6 months and the final report upon completion. The PI should also notify the AAERC ahead of any amendments or modifications in the protocol or premature suspension or termination of the study.

Signature: _____

CHAIRPERSON
Name: Prof. Getachew Tilahun

Signature: _____

SECRETARY
Name: Dr. Liya Wassie

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636

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Observational / Interventions Research Ethics Committee

Edessa Negera Gobena
Research Degree Student
CR / ITD
LSHTM

22 April 2013

Dear Mr. Gobena,

Study Title: **The Immunopathology of Erythema Nodosum Leprosum (ImpaENL)**
LSHTM ethics ref: **6391**

Thank you for your email of 20 April 2013, responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
LSHTM ethics application	V2	20/04/2013
SOPs		
Information sheet		20/04/2013
Consent form		20/04/2013

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form. All studies are also required to notify the ethics committee of any serious adverse events which occur during the project via form E4. At the end of the study, please notify the committee via form E5.

Yours sincerely,

Professor John DH Porter
Chair
ethics@lshtm.ac.uk
http://intra.lshtm.ac.uk/management/committees/ethics/

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The Federal Democratic Republic of Ethiopia
Ministry of Science and Technology

ቁጥር 3.10/450/06
Ref. No.
ቀን 12/03/06
Date

To: Aramuer Hansen Research Institute (AHRI)
Addis Ababa

Re: The Immunopathaology of Erythema Nodosum Leprosum
Dear sir/Mr./s/Dr.

The National Research Ethics Review committee (NRERC) has reviewed the aforementioned project protocol in an expedited manner. We are writing to advise you that NRERC has granted

Full Approval

To the above named project, for a period of **one year (November 20, 2013- November 19, 2014).** All your most recently submitted documents have been approved for use in this study. The study should comply with the standard international and national scientific and ethical guidelines. Any change to the approved protocol or consent material must be reviewed and approved through the amendment process prior to its implementation. In addition, any adverse or unanticipated events should be reported within 24-48 hours to the NRERC. Please ensure that you submit progress report once in a four month and annual renewal application 30 days prior to the expiry date.

We, therefore, request your esteemed organization to ensure the commencement and conduct of the study accordingly and wish for the successful completion of the project.

With regards,

Yohannes Sitotaw

Secretary of NRERC

Cc Mr Edessa Negera (PI)

The Federal Democratic Republic of Ethiopia * Ministry of Science and Technology *

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You may Contact

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Ref No ለጥ/ህ/ህ/ቁ/6449/2024 ዓ/ም

Date 26/07/13

To ALERT /AHRI Ethical I eview Committee
ALERT

Subject: - Support Lette

The research proposal entitl d "**The Immuno pathology of ENL**" is going to be conducted by Edessa Negera

This is therefore to notify th committee that we are willing to allow the research to be conducted at ALERT ospital and extend our support as the request of the investigator after the propos l gain approval from ALERT/AHRI Ethical review committee.

Sincerely Yours,

Dr. Lidiya Tefera
Chief Clinical
Service Officer

Cc:

Chief Executive Offic r

Material Transfer Agreement
Signature page

For Recipient:

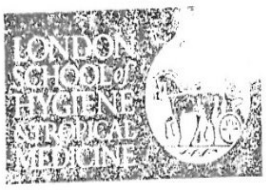
Recipient's Investigator
Edessa Negera Gobena

Signature

Date 24/8/2013

Duly Authorized
Signature/ Stamp

Date 24/8/2013



Mailing Address for Material:
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Keppel Street, London, UK
WC1E 7HT
Tel: +44 (0) 2079272642
Fax: +44 20 7637 4314

Mailing Address for Notices:
London School of Hygiene and Tropical Medicine, Keppel Street, London, UK
WC1E 7HT
Tel:+44 (0) 20 7927 2601
Fax: +44 (0) 20 7927 2739

For Provider

Provider's Investigator
Kidist Bobosha

Signature

Date ----------

Duly Authorized
Signature/stamp

Date ---------- 22/8/13

Mailing Address:
Armauer Hansen Research Institute
Jimma Road, Addis Ababa

Mailing Address for Notices:
Armauer Hansen Research Institute
Jimma Road, Addis Ababa

Appendix 2: Information Sheet and Informed Consent

- Information sheet (English version)
- Informed-consent (English version)
- Information sheet (Amharic version)
- Informed-consent (Amharic version)

INFORMATION SHEET

London School of Hygiene
and Tropical Medicine
Keppel Street, London WC1E
7HT
UK
Tel. +44(0)2076127863

All Africa Leprosy,
Tuberculosis and
Rehabilitation Training
centre
Ayer Tena road
PO Box 165. Addis Ababa.
Ethiopia
Tel. +251 113211334

1. **Study title: The Immunopathology of Erythema Nodosum Leprosum (EmpaENL)**

2. **Invitation paragraph**

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and to talk to others about the study, if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

3. **The purpose of the study**

The underlying cause of ENL is poorly understood. Understanding the cause of this condition and identifying risk factors which contribute to its development is useful for developing strategies to reduce the morbidity and mortality caused by ENL. This knowledge is crucial for the development of new and better treatments as well as proper management of ENL patients through improving diagnosis and treatment. In

the project we are investigating the immunological changes that occur when lepromatous leprosy (LL) patients develop ENL reactions. The information obtained from this study will also be used to develop better patient treatment.

4. Why you have been chosen?

You are invited to participate in this study as a leprosy patient with ENL or without ENL. We want to know the immunological changes that take place during the course of ENL and when LL patients undergo ENL reaction. In this study 45 patients with ENL and 30 LL patients without ENL will participate.

5. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign consent form. You are free to withdraw at any time, without giving a reason. A decision not to take part or to withdraw at any time, will not affect the standard of care you receive.

6. What will happen to me if I take part?

Your role in the study:

If you agree to participate, the doctor in charge to treat your case (leprosy) will ask you some questions which you are expected to answer. These are: history of treatment related to your current case, symptoms and time of the onset of the disease. In addition you will be asked your age, current address and educational status. You will be asked these questions as a normal treatment procedure even if you will not take part in this study.

Then you will be asked to give 50ml blood (equivalent to about 10 tea spoons or one and half of egg cup) which will be collected by inserting a small needle into a vein in your arm and one 4mm and 6mm skin biopsy samples from the diseased, part after receiving an anaesthetic injection. You will be asked to return after 90 days (12 weeks) and 180 days (24 weeks) to donate the same amount of blood. Skin biopsies are not required on 12 week. At the same time you will be given antileprosy treatment free of charge according to the national treatment guideline for leprosy (LL or ENL) irrespective of this study by your doctor.

7. What I have to do?

You will be expected to attend the initial donation (blood and biopsy) and subsequent clinical investigation, blood and biopsy donations. If, for any reason you are unable to attend one or more of the appointments please let us know in advance.

8. What are the possible disadvantages and risks of taking this part?

By participating in this research project, you may feel that it is inconvenient that you have to come to the hospital 3 times but you are normally expected to be followed for 1 year treatment for your sickness (leprosy) and come to the hospital even if you do not take part in this study.

There is no major risk in participating in this research, but the minor bleeding that may occur during blood collection will be avoided, as the procedure is carried out by trained experienced health professionals on the standard good clinical practice.

9. What are the possible benefits of taking part?

If you participate in this research, you may not get any direct benefit but anything regarding your leprosy treatment case will be followed with greater care based on your laboratory results. In addition, your participation is likely to help us in understanding the disease process of ENL which may benefit future patients by developing treatments for ENL reactions.

10. Incentives

You will not be provided any incentive to take part in this research. However, you will be given 100 ETB for lunch and your travel expenses during participation in the project during each visit of the hospital up on the follow-up appointment given to you for a maximum of three times.

11. What happens when the research study stops?

On completing the study no further participation is required.

12. What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this issue is given in part 2 in this booklet.

13. Will my taking part in the study is kept confidential?

The information that we collect from this research project will be kept confidential. Information about you that will be collected from the study will be stored in a file, which will not have your name on it, but a code number assigned to it. Which number belongs to which name will be kept separately in a password protected data management file and it will not be revealed to anyone except the principal investigator and your treating physician. Your personal information will not be disclosed even during the reporting of the findings. Reports will be written and disclosed anonymously.

14. Contact details

Study coordinator and Principal investigator

Edessa Negera Gobena

Mobile: 0911880085

AHRI, Ethiopia

Study clinician

Dr. Shimelis Nigusie

Mobile no: 0911642060

ALERT Hospital, Ethiopia

Local Study Manager (Local Supervisor)

Dr. Abraham Aseffa

Tel. +251 113211334

AHRI, Ethiopia

This completes part 1 of the information sheet. If the information in part 1 has interested you and you are considering participation, please continue to read the additional information in part 2 before making any decision.

Part 2

15. What if relevant information becomes available?

If the study is stopped for any reason, you will be told why and your care will not be affected by the discontinuation of the study.

16. What will happen if I don't want to carry on with the study?

If you withdraw from the study, we will remove and destroy all your identifiable samples but we will need to use the data collected up to your withdrawal.

17. What if something goes wrong?

The London School of Hygiene and Tropical Medicine holds insurance policies which apply to this study. If you are harmed due to someone's negligence, then you many have grounds for legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the local project manager (Abraham Aseffa), project coordinator (Edessa Negera) or the study physician (Shimelis Nigissie).

18. What will happen to any samples I give?

As already described, during the laboratory analysis we will use your given code not your name for your sample. The majority of the samples are immediately used but some will be stored after semi-processed for further immunological assays. Any unused samples will be destroyed within 5 years. The data collected will be written and published in peer reviewed scientific journals. A list of publications can be accessed on the LSHTM website. Biopsy samples will be transferred to collaborating laboratories abroad (LSHTM) for immunohistochemistry since this method needs both expertise and advanced facilities which are not available in the country.

19. What will happen to the results of the study?

Data from this study will be analysed and published in scientific journals but your identity will not be revealed. Data will also be presented at seminars at national and international meetings. No information containing your name will be disclosed.

20. Who is organizing and funding the project

The research is funded by the Hospital and Homes of St Giles and sponsored by the London School of Hygiene and Tropical Medicine

21. Who has reviewed the study?

This study was given a favourable ethical opinion by the AHRI/ALERT Research Ethics committee and by the London School of Hygiene and Tropical Medicine Research Ethics committee.

You will be given a copy of the information sheet and a signed consent form to keep.

LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

AHRI ALERT
Armauer Hansen Research Institute

INFORMED CONSENT FORM

Full Title of Project: The Immunopathology of Erythema Nodosum Leprosum (Empa ENL)

Name of the principal investigator: Edessa Negera Gobena

Please put Initial box

1.I Confirm that I have read and understand the participant information sheet dated .....….. (Version .....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered fully.	
2. I understand that my participation is voluntary and I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
2.I understand that sections of my medical notes and data collected during the study may be looked at by responsible individuals from the London School of Hygiene & Tropical Medicine, AHRI, regulatory authorities or from ALERT hospital, where it is relevant to my taking part in this research. I give permission for these individuals to access my records. I agreed that my samples can be used for the intended research and for any investigations related to it.	
3.I agree for the tissue samples to be transported and used at London School of Hygiene & Tropical Medicine for the intended research project and related mentioned above.	
4.I agree to take part in the above study.	

Name of Participant *(printed)*	Signature/Thumbprint	Date
Name of Person taking consent	Signature	Date
Principal Investigator	Signature	Date

The participant is unable to sign. As a witness, I confirm that all the information about the study was given and the participant consented to taking part.

Name of Impartial Witness *(if required)*	Signature	Date

1 copy for participant; 1 copy for Principal Investigator; 1 copy to be kept with hospital notes

Information sheet: Amharic version

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የአለርት ሪፈራል ሆስፒታል እና የአርማወር ምርምር ኢንስቲቲዩት

<u>በጥናቱ እንድሳተፉ ስለመጋበዝ</u>

በዚህ ጥናት ላይ እንዲሳተፉ እንጋብዘዎታለን ነገርግን በጥናቱ ከመሳተፍዎ በፊት የጥናቱ አላማና አስፈላጊነትን በቅድሚያ መረዳት ያስፈልገዎታል፡፡ በዚሁ መሠረት ይህ መረጃ ሰጪ ጥራዝ በሁለት ክፍሎች የተዘጋጀ ነው፡፡ የመጀመሪያው ክፍል የጥናቱ ዓለማና የእርሶ ለጥናቱ አስፈላጊነት በአጭሩ ይገልፃል፡፡ ክፍል ሁለት ስለጥናቱ አሠራርና አካሄድ በጥልቀት ያብራራል፡፡

<u>ክፍል አንድ</u>

<u>የጥናቱ ዓላማ</u>

አንዳንድ የሥጋ ደዌ በሽተኞች ኢሪተማ ኖዶሱም ለኘሮሱም በሚባል በሽታ ይጠቃሉ ፡፡ ይህ በሽታ አንዱ የሥጋ ደዌ አይነት ሲሆን ጠቅላላ የሰውነት ክፍሎችን በተለይም አይንና ነርቭን የሚያጠቃና ከፍተኛ ትኩሳትንና የመሳሰሉትን ያስከትላል፡፡ በዚህ በሽታ የተጠቁ የስጋ ደዌ ህመምተኞች ከፍተኛ የህመም ስሜት ይኖራቸዋል፡፡ በአንጻሩ አንዳንድ የሥጋ ደዌ በሽተኞች ለምንና በምን ምክንያት በዚህ በሽታ እንደሚጠቁ በቂ ጥናት አልተደረገም፡፡ ስለዚህ የዚህ ጥናት ዋና ዓላማ የበሽታውን መንስኤና ምልክቶቹን ማጥናት ነው፡፡ የበሽታውን መንስኤና ምክንያቶች ማወቅ ጥሩ የህክምና ስልት ለማዘጋጀትና የሥጋ ደዌ በሽተኞች በዚህ በሽታ እንዳይጠቁ ቀድሞ በመተንበይ መከላከል ያስችላል፡፡

<u>እርሶ ለምን በዚህ ጥናት እንዲሳተፉ ተመረጡ?</u>

እርሶ በዚህ ጥናት ላይ እንዲሳተፉ የተመረጡበት ምክንያት የሥጋ ደዌ ህመምተኛ በመሆንዎና በጥናቱ ሊካተቱ የሚችሉት የሥጋ ደዌ ህመምተኞች ብቻ በመሆናቸው ነው፡፡

<u>በዚህ ጥናት ላይ ለመሳተፍ የግድ ያስፈልጋል?</u>

በጥናቱ ላይ ለመሳተፍ የግድ አያስፈልግም፤ በፍላጎት ላይ ብቻ የተመሠረተ ነው፡፡ በጥናቱ ላይ ለመሳተፍ ከወሰኑ ይህ መረጃና መስማማትዎን የሚገልጽ ቅጽ ይሰጠዎታል፡፡ መረጃውን ካነበቡና የሚጠይቁት ጥያቄ ካለም በመጠየቅ በሚገባ ከተረዱ በኃላ መስማማትዎን ይገልጻሉ፡፡

<u>በጥናቱ ላይ ከተሳተፍኩ ከእኔ ምን ይፈለጋል?</u>

<u>በጥናቱ ላይ ለመሳተፍ ከተስማሙ</u>

1. መስማማተዎን በፊርማ ያረጋግጣሉ
2. ስለ በሽታዎ አንዳንድ ጥያቄዎች ይጠየቃሉ፡፡ ይህ ጥያቄ በመደበኛ የሕክምና ጊዜ የሚጠይቅ በመሆኑ የዚህ ጥናት ብቻ ጥያቄ አይደለም፡፡
3. ስለ በሽታዎ ለማጥናት 50 ሚሊ ሊትር ደም (ግማሽ የሻይ ኩባያ ያህል) እንዲለግሱ ይጠየቃሉ፡፡ በተጨማሪም በበሽታው ምክንያት ከቆሰለው የቆዳዎ ክፍል 6 ሚሊሜትር እና 4 ሚሊሜትር ቁራጭ (ባዮፕሲ) ይወሰድና ይጠናል፡፡ አወሳሰዱ በሰለጠኑ ባለሙያዎች ስለሆነ የሚከሰት ስጋት አይኖርም፡፡ ደምና የባዮፕሲ ልገሳ በአጠቃላይ ለሶስት ጊዜ በየ12 ሳምንት ይሆናል፡፡

<u>ከእርስዎ ምን ይጠበቃል?</u>

በጥናቱ ለመሳተፍ ከተስማሙ ከላይ የተገለፀውን የደምና የቆዳ ቁራጭ (ባዮፕሲ) ለሶስት ጊዜ እንዲለግሱ ይጠበቃል፡፡ በቀጠሮው ቀን መምጣት የማይችሉ ወይም የማይፈልጉ ከሆነ ቀድመው እንዲያሳውቁን በአክብሮ ትብብርዎን እንጠይቃለን፡፡

<u>በጥናቱ ላይ ቢሳተፉ ጥቅማ ጥቅም አገኛለሁኝን?</u>

በዚህ ጥናት ላይ በመሳተፍዎ የተለየ ጥቅም በግል አያገኙም፡፡ ነገርግን የትራንስፖርትና የምሳ ወጭዎ ይሸፈንልዎታል፡፡ በተጨማሪም በጥናቱ ውጤት መሰረት ለበሽታዎ ልዩ ክትትል የሚያስፈልግ ከሆነ በሀኪም ክትትል ይደርግለዎታል፡፡ ከሁሉም በላይ ግን በዚህ ጥናት የሚገኘው ውጤት ስለበሽታው ጥሩ ግንዛቤ እንዲኖረንና በሽታውን ለመቆጣጠር ጠቃሚ መረጃ ስለሚሰጠን ለህብረተሰባችን የሚያስገኘው ጥቅም ከፍተኛ ነው፡፡ ከሩቅ ቦታ (ከፍለሀገር) የሚመጡ ከሆነ የትራንስፖርት፣ የመኝታና የምግብ ወጭዎ ይሸፈናል፡፡ በተጨማሪም ቀጠሮዎ ለበሽታዎ ክትትል ወደ አለርት ሆስፒታል ከሚመጡበት ቀን ጋር አንዲጣጣም ይደረጋል፡፡

ክፍል ሁለት

ጥናቱ ድንገት ቢቋረጥ በበሽታዬ ህክምና ላይ ተጽዕኖ ይኖረዋልን?

አይኖረውም፡፡ ጥናቱ ቢያልቅም ወይም ቢቋረጥ የርስዎ የህክምና እርዳታና ከትትል እንደማንኛውም ስጋ ደዌ ህመምተኛ ይቀጥላል፡፡

ከጥናቱ እራሴን ማግለል እችላለሁ?

አዎ ይችላሉ፡፡ ከጥናቱ በፈለጉት ጊዜና ሰዓት ያለምንም ቅድመ ሁኔቴ ማቋረጥ ይችላሉ፡፡ እራስዎን ከጥናቱ በማግለልዎ ምክንያት ለህመምዎ የህክምና እርዳታ ከማግኘት አያግድም፡፡ እንደማንኛውም ታካሚ አስፈላጊውን የህክምና እርዳታ ያገኛሉ፡፡

እኔ የምለግሰው ናሙና ምን ይሆናል?

የርስዎ ናሙና የተለያ ቁጥር ይሰጠዋል፡፡ በናሙናው ላይ የርስዎ ስም አይጻፍም፡፡ አብዛኛው ናሙና ወዲውኑ ስራ ላይ ይውላል፡፡ ጥቂት ናሙና ግን ለመጠባበቂያነትና ለከፍተኛ ምርመራ ወደ ለንደን የሀይጅንና ትሮፒካል ሜዲስን ት/ቤት ይላካል፡፡ ናሙናው በሚላክበት ጊዜ በልዩ ኮድ (መለያ ቁጥር) ብቻ ይሆናል፡፡ በጥናት የሚገኘው መረጃ በህትመት መልክ ለጤና ባለሙያና ለሳይንትስቶች ይደርሳል፡፡ ውጤት በጅምላ በሚገለጽበት ጊዜ የማንንም ውጤት አይወክልም፡፡

ጥናቱ ሲያልቅ ከእኔ ምን ይጠበቃል?

ምንም አይጠበቅም፡፡ ጥናቱ ካለቀ የርስዎ መሳተፍ ያበቃል፡፡ ጥናቱ በሚያልቅበት ጊዜም ይነገርዎታል፡፡

ቅሬታ ቢኖረኝ ምን አድርጋለሁ?

ከዚህ ጥናት ጋር በተያዘ ማንኛውንም ቅሬታ ለጥናቱ ማናጀር (ዶ/ር አብርሃም አሰፋ) ወይም ለጥናቱ ተመራማሪ (ለኤዬሳ ነገራ) ማመልከት ይችላሉ፡፡

በዚህ ጥናት መሳተፍ በሚስጥር ይያዛልን?

አዎን ለዚህ ጥናት የሚሰበሰበው ናሙና እና የናሙና ውጤት በሚስጥር ይያዛል፡፡ ስለ እርስዎ የሚገልጽ ማንኛውም ነገር በናሙናም ሆነ በውጤቱ ላይ አይጻፍም፡፡ ውጤት ሲገለጽ ስም አልባ ይሆናል፡፡ ለእያንዳንዱ ናሙና ልዩ መለያ ቁጥር ወይም ምልክት ይሰጠዋል፡፡ የትኛው ቁጥር የማን እንደሆነ ዋና ተመራማሪው ብቻ ያውቃል፡፡ ስለ ናሙናዎ ውጤት ከዋና ተመራማሪው በተጨማሪ እርሶን የሚከታተለው ሀኪምም ሊያውቀው ይችላል፡፡

<u>ይህ ጥናት ተቀባይነትን አግኝቷል?</u>

ይህ ጥናት በአህሪ/አለርት የስነ-ምግባር ኮሚቴና በለንደን የሃይጅንና ትሮፒካል ሜድስን ት/ቤት የስነ-ምግባር ኮሚቴ ተገምግሞ ተቀባይነትን አገኝቶ ፀድቋል፡፡

<u>ተጨማሪ መረጃ ከፈለጉ የሚከተሉትን ባለሚያዎች ማነጋገር ይችላሉ</u>

1. የጥናቱ አስተባባሪና ዋና ተመራማሪ ኢዴሳ ነገራ ጎበና ስልክ ቁ. 0911880085
2. የጥናቱ ሀኪም ዶ/ር ሺመልስ ንጉሴ ስልክ ቁ. 0911642060
3. የጥናቱ ማናጀር ዶ/ር አብርሃም አሰፋ ስልክ ቁ. 0113211334

Informed consent form: Amharic version

የኢሪተማ ኖዶሱም ለኘሮሱም ክስተትና የበሽታውን ባህሪይ ማጥናት

የለንደን የሃይጅን እና የትሮፒካል ሜዲሲን ት/ቤት፣ ዩናይትድ ኪንግደም
የአለርት ሪፈራል ሆስፒታል እና የአርማወር ምርምር ኢንስቲቲዩት

የስምምነት መግለጫ ቅጽ

የጥናቱ ተማራማሪ፡ ኤዴሳ ነገራ ጎበና

		እባክዎት ሳጥኑ ውስጥ ምልክት ያድርጉ
1.	ከዚህ በለይ ለተጠቀሰው ጥናት የ----------ቀን (ቁጥር----------) የመረጃ ወረቀት አንብቤ ጉዳዩን መረዳቴን አረጋግጣለሁ፡፡	☐
2.	ተሳትፎዬ በፈቃደኝነት መሆኑን እና በማንኛውም ጊዜ ምንም አይነት ምክንያት ማቅረብ ሳያስፈልገኝ፤ የህክምናዬ ክብካቤ ወይንም ህጋዊ መብቴ ሳይነካ ማቋረጥ እንደምችል ተረድቻለሁ፡፡	☐
3.	ከእኔ የሚወሰደው ናሙና እና የጤና መረጃ በተመራማሪዎቹና በሽታዬን በሚከታተለው ሀኪም መታየት እንደሚችልና የለገስኩት ናሙና ከላይ ለተገለጻው ጥናት እንዲውል ፈቅጃለሁ፡፡	☐
4.	እኔ የምለግሰው ናሙና (የቆዳ ቁራጭ) ወደ ለንደን የሀይጅንና ትሮፒካል ሜድስት ት/ቤት ተወስዶ ተጨማሪ ምርመራ እንዲደረግበት ፈቅጃለሁ፡፡	☐
5.	በዚህ ጥናት ለመሳተፍ በሙሉ እውቅናዬ የተስማማሁ መሆኔን አረጋግጣለሁ፡፡	☐

_____

_____ _____

የተሳታፊ ስም ፊርማ ቀን

_____

_____ _____

የውል ተቀባይ ስም ፊርማ ቀን

_____

_____ _____

የጥናቱ ዋና ተመራማሪ ስም ፊርማ ቀን

ይህ በጥናቱ የሚሳተፈው ሰው መፈረም ስለማይችል ከላይ የተዘረዘሩት መረጃዎች ለተሳታፊው የተሰጡና ተሳታፊውም ለመሳተፍ መስማማቱን ገለልተኛ ታዛቢ በመሆን አረጋግጣለሁ፡፡

_____

_____ _____

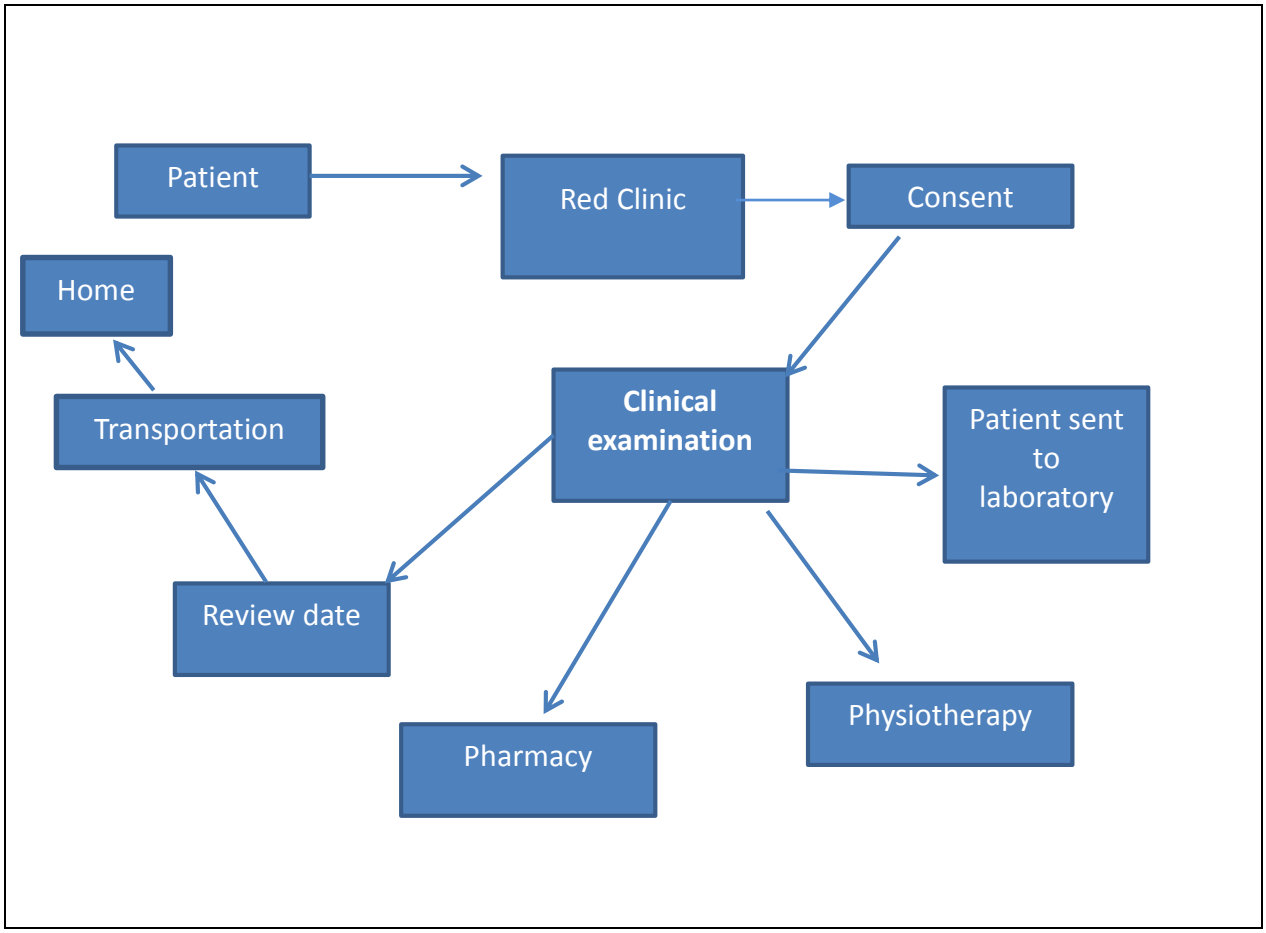
የገለልተኛ ታዛቢ ስም ፊርማ ቀን

Appendix 3: Patient flow diagram and Clinical data collection sheet

- Patient flow diagram
- Clinical data collection sheet and enrolment criteria
- Enrolment criteria
- ENL patients review date (appointment date) form
- Study participant indntification card
- Clinical Data Collection sheet
- Muscle testing form
- Sensory testing form
- Histopathology data sheet

Patient flow diagram

Title of the study: The Immunopathology of Erythema Nodosum Leprosum



Patient flow and Recruitment method (summary).

1. Patients identified at Red Medical Clinic
2. Patients informed about the study and recruited with consent.
3. Fully history and examination by the study physician
4. Patient sent for laboratory investigation
5. Patient sent to physiotherapy for sensory and voluntary muscle testing
6. Patient reviewed by the study physician
7. Patient referred to pharmacist for treatment
8. Patient given review date
9. Transportation and lunch compensated (depending on the address of the patient)

Clinical data collection sheet and enrolment criteria

Title of the study: The Immunopathology of Erythema Nodosum Leprosum

ALERT No. ----------AHRI No. ---------- Study number ----------

Entry Criteria: Patient history; Patient examination; Consent form

Day 0: 6mm + 4mm Skin punch Biopsy: ½ (6mm) frozen storage (-80^{o}c) and ½(6mm) in 10% formalin (room T) and 4mm in RNAlater (-20 ^{o}c) 40-50mL blood sample for (ELISA, Flow cytometry, Circulating IC, mRNA RT-PCR)
Week 12: 50mL blood sample for (ELISA, Flow cytometry, Circulating IC)
Week 24: 6mm + 4mm Skin punch Biopsy: ½ (6mm) frozen storage (-80^{o}c) and ½(6mm) in 10% formalin (room T) and 4mm in RNAlater (-20 ^{o}c) 40-50mL blood sample for (ELISA, Flow cytometry, Circulating IC, mRNA RT-PCR)

NB: please add 2mL blood to PAXgene Blood RNA tubes and store at (-20 ^{o}c.

Clinical data required: Full clinical investigation report on day 0, 12 weeks and 24 weeks

Date the patient enrolled in the study dd / mm /yy E.C.

Date patient returned for follow up

Week 12: dd / mm / yy

Week 24 : dd / mm / yy

Physician in charge: (Name and signature) ----------.

Nurse in Charge ‘’’ ----------.

PI in charge ‘’ ----------.

ENL patients review date (appointment date)

Please use Ethiopian Calendar (ddmmyy)

No	Name	Study code	Steroid completion date	First visit (date)	Second review date	3rd review date	Address (contact phone)

LL patients review date (appointment date)

Please use Ethiopian Calendar (ddmmyy)

No	Name	Study code	First visit (date)	Second review date	3rd review date	Address (contact phone)

Study participant indntification card. Project code 021

አለርት ሆስፒታል፣ ሬድ ሜድካል ክሊኒክ
የቀጠሮ ካርድ (ENL ፣LL)
ስም_____**መ.ቁ.**_____

የቀጠሮ ሳምንት	የቀጠሮ ቀን	የታየበት ቀን	ምርመራ
4ኛ ሳምንት			
8ኛ ሳምንት			
12ኛ ሳምንት			
16ኛ ሳምንት			
20ኛ ሳምንት			
24ኛ ሳምንት			
28ኛ ሳምንት			

አለርት ሆስፒታል፣ ሬድ ሜድካል ክሊኒክ
የቀጠሮ ካርድ (ENL ፣LL)
ስም_____**መ.ቁ.**_____

የቀጠሮ ሳምንት	የቀጠሮ ቀን	የታየበት ቀን	ምርመራ
4ኛ ሳምንት			
8ኛ ሳምንት			
12ኛ ሳምንት			
16ኛ ሳምንት			
20ኛ ሳምንት			
24ኛ ሳምንት			
28ኛ ሳምንት			

አለርት ሆስፒታል፣ ሬድ ሜድካል ክሊኒክ
የቀጠሮ ካርድ (ENL ፣LL)
ስም_____**መ.ቁ.**_____

የቀጠሮ ሳምንት	የቀጠሮ ቀን	የታየበት ቀን	ምርመራ
4ኛ ሳምንት			
8ኛ ሳምንት			
12ኛ ሳምንት			
16ኛ ሳምንት			
20ኛ ሳምንት			
24ኛ ሳምንት			
28ኛ ሳምንት			

አለርት ሆስፒታል፣ ሬድ ሜድካል ክሊኒክ
የቀጠሮ ካርድ (ENL ፣LL)
ስም_____**መ.ቁ.**_____

የቀጠሮ ሳምንት	የቀጠሮ ቀን	የታየበት ቀን	ምርመራ
4ኛ ሳምንት			
8ኛ ሳምንት			
12ኛ ሳምንት			
16ኛ ሳምንት			
20ኛ ሳምንት			
24ኛ ሳምንት			
28ኛ ሳምንት			

Clinical Data Collection sheet

Form 1 Patient ID----------

PATIENT DETAILS				PHYSICIAN			
Patient ID		New ENL case □	Recurrent ENL case □	Chronic ENL case □			
Date	__/__/__						
Gender	Male □	Female □	Post menopause Yes □ No □	Date of birth	__/__/__	Age	
LEPROSY DETAILS							
Date of diagnosis of leprosy	__/__/__			Ridley-Jopling	BB □	BL □	LL □
Bacterial index at diagnosis		Mean BI	____	Highest BI at any single site	____		
Current BI		Mean BI	____	Highest BI at any single site	____		
MB MDT Start date	__/__/__			MB MDT Completed date	__/__/__		
Presented with reaction at leprosy diagnosis	NO □	YES □	TYPE 1 □	ENL □	NEURITIS □		
Date of onset of most recent T1R or neuritis	__/__/__			No previous T1R or neuritis □			
Other illnesses		TB □	Diabetes □	HIV □	Other (specify)		
ENL DETAILS							
Duration of current symptoms (DAYS)	____	Number of previous ENL episodes (if recurrent)		0	1-4	5-9	≥10

Current ENL treatment (drug and dose)		Date started	__/__/__	<u>Previous ENL treatments:</u> Prednisolone Clofazimine Thalidomide Pentoxifylline Aspirin (or NSAID) Other (please state)			
Current symptoms	Fever □	Skin lesions □	Other localised swelling (not skin) □ _____	Peripheral oedema □	Insomnia □	Anorexia □	
	Weight loss □	Nasal stuffiness□	Depression □	Malaise □	Epistaxis □	Joint swelling □	
Pain symptoms	Skin □	Bone □	Digits □	Testes □	Eyes □		
	Muscles □	Lymph nodes □	Nerves □	Joints □			
Where is pain worse?							
Nerve symptoms	Pain □	Weakness □	Reduced sensation □	Paraesthesia □		Hyperaesthesia □	
	New NFI □	Old NFI □					
EXAMINATION			Urinalysis	Protein □	Blood □	EHF Score	___ _
Temperature	___°C		Weight (kg)	_____	BP	/	
Type ENL skin lesions	Papules □	Nodules □	Vesicles □	Bullae □	Pustules □		
	Plaques □	Ulcerated □	Necrotic □	Sub-cutaneous nodules □	EM like lesions □	Scar due to ENL □	
Size of largest lesion (mm)			Describe unusual skin lesions such as EM-like				

Number of ENL skin lesions	5 or less □	6-10 □	11-20 □	21-50 □	>50 □		
Number of ulcerated/necrotic lesions							
Location of ENL skin lesions	Head/neck□	Trunk □	Upper limbs □	Lower limbs □			
Oedema	Face □	Hands □	Lower limbs □	Localised □	Specify:		
Dactylitis	Yes □	No □					
Arthritis	Yes □	No □	Large joints □	Small joints □			
Orchitis	Left □	Right □					
Eyes							
Lagophthalmos	Left □	Right □					
Conjunctivitis	Left □	Right □					
Anterior uveitis	Left □	Right □					
Scleritis	Left □	Right □					
Episcleritis	Left □	Right □					
Visual acuity	Left	/	Right	/			
Lymphadenitis	Yes □	No □	Site/s:				
Liver tenderness	Yes □	No □					
Rhinitis	Yes □	No □					
Other signs associated with ENL	Yes □	No □	Specify:				

Patient ID----------

Form 2: For visits at weeks 12 and 24 after enrolment

PATIENT DETAILS				PHYSICIAN			
Patient ID		Date	__/__/__				
ENL DETAILS							
Current symptoms	No symptoms of ENL □	Fever □ Skin lesions □	Other localised swelling (not skin) □ _____	Peripheral oedema □	Insomnia □	Anorexia □	
	Weight loss □	Nasal stuffiness □	Depression □	Malaise □	Epistaxis □	Joint swelling □	
Pain symptoms	Skin □	Bone □	Digits □	Testes □	Eyes □		
	Muscles □	Lymph nodes □	Nerves □	Joints □			
Where is pain worse?							
Nerve symptoms	Pain □	Weakness □	Reduced sensation □	Paraesthesia □		Hyperaesthesia □	
	New NFI □	Old NFI □					
EXAMINATION			Urinalysis	Protein □	Blood □	EHF Score	____
Temperature	___°C		Weight (kg)	_____	BP	/	
Type ENL skin lesions	Papules □	Nodules □	Vesicles □	Bullae □	Pustules □		
	Plaques □	Ulcerated □	Necrotic □	Sub-cutaneous nodules □	EM like lesions □	Scar due to ENL □	
Size of largest lesion (mm)			Describe unusual skin lesions such as EM-like				

Number of ENL skin lesions	5 or less □	6-10 □	11-20 □	21-50 □	>50 □		
Number of ulcerated/necrotic lesions							
Location of ENL skin lesions	Head/neck □	Trunk □	Upper limbs □	Lower limbs □			
Oedema	Face □	Hands □	Lower limbs □	Localised □	Specify:		
Dactylitis	Yes □	No □					
Arthritis	Yes □	No □	Large joints □	Small joints □			
Orchitis	Left □	Right □					
Eyes							
Lagophthalmos	Left □	Right □					
Conjunctivitis	Left □	Right □					
Anterior uveitis	Left □	Right □					
Scleritis	Left □	Right □					
Episcleritis	Left □	Right □					
Visual acuity	Left	/	Right	/			
Lymphadenitis	Yes □	No □	Site/s:				
Liver tenderness	Yes □	No □					
Rhinitis	Yes □	No □					
Other signs associated with ENL	Yes □	No □	Specify:				

Name: _____

Right							MUSCLE TESTING							Left
							Date							
							FACIAL							
							Eyeclosure							
							ULNAR							
							Abd. 5th. finger							
							ULNAR/MEDIAN							
							Abd. index finger							
							MEDIAN							
							Abduction thumb							
							Opposition thumb							
							RADIAL							
							Wristextension							
							COMM. PERONEAL							
							Doraiflex. foot							
							Eversion foot							
							Sign.							

First assessment weakness/ paralysis in red. Follow-up assessments only deterioration in red:

Comments: (Include duration of nerve damage)

Date_____Sign_____

Blink yes / no

Lagophthalmos: Bell + / -

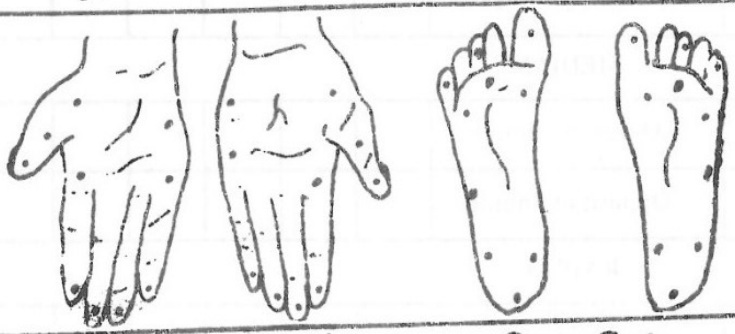
Footwear:

Date_____Sign_____

Blink yes / no

Lagophthalmos: Bell + / -

Footwear:



Date_____Sign_____

Blink yes / no

Lagophthalmos: Bell + / -

Footwear:

Date_____Sign_____

Blink yes / no

Lagophthalmos: Bell + / -

Immunopathology of ENL; Histopathology data sheet

AHRI No	PMN	Lymphocytes	Foamy Histiocytes	Multinucleated Giant cells	Eosinophils & must cells	Granuloma	PMN inf. pattern	Vasculitis	Panniculitis	Lobular vasculitis	Necrosis	Swelling of Endothelial cells	Oedema of vessel walls

Immunopathology of ENL (patient follow up form) ENL

no	Code	ENL type at recruitment (New, chronic, recurrent)	Treated before with steroid (Yes, NO)	MDT status during sampling (Yes, No)			Steroid dose on sampling date (put the dose when each samples taken)			Remark
				1st sample	2nd sample	3rd sample	1st sample	2nd sample	3rd sample	

Immunopathology of ENL (patient follow up form) **LL**

no	Code	LL type at recruitment (new, defaulter, relapse)	MDT status during sampling (Yes, No)			Any other drugs given including steroids			Remark
			1st sample	2nd sample	3rd sample	1st sample	2nd sample	3rd sample	

Blood and Biopsy Form (Project Code P032/12)

The Immunopathology of ENL

NO	Date (ddmmyy)	Study no	ALERT No	AHRI No	sex	Age	Blood volume	Biopsy body site	Any remark	Name and signature of person in charge

Appendix 4: Laboratory Standard Operating Procedures (Sops)

- Protocol 1: PBMC isolation
- Protocol 2: PBMC freezing protocol
- Protocol 2: PBMCs thawing
- Protocol 4: Reagents preparation
- Protocol 5: FACS staining
- Protocol 6. RNA Extraction
- Protocol 7: cDNA synthesis for real-time two-step RT-PCR
- Protocol 8: Real-Time PCR

Protocol 1: PBMC isolation (perform under sterile condition)

Method: Ficoll-paque density gradient centrifugation

Status: optimized for the intended plan in this proposa**l**

PBMC isolation (sing Leuocosep tubes (cat. no 227 289)

1. Warm-up separation medium (Ficoll-Paque Plus) to RT
2. Fill the Leucosep® tube with separation medium (15mL Ficoll-Paque)
3. centrifuge for 1 minute at 900 rpm at RT
4. Fill with anticoagulated blood (25mL)
5. Centrifuge 25 minutes at 1800 rpm **(Break Off)**
6. Collect the plasma using pasteur pipette into cryotube and keep at -20
7. Collect the PBMC using Pasteur pipette into another centrifuge tube
8. Wash the enriched cell fraction (lymphocytes / PBMC's) with 10 mL of phosphate-buffered saline (PBS), subsequently centrifuge for 10 minutes at 1800 (**Break off)**
9. Repeat washing at 1520 rpm for 10 minutes (break on low)
10. Re-suspend cells in cRPMI **Note: from here after use ice**
11. Count the cells
12. Make 20 million cells /mL in cRPMI
13. Proceed to Freezing

Protocol 2: PBMC freezing protocol

Reagents

A. Complete medium (cRPMI)
   1% Pen + 1% strep + 10% FBS +88% RPMI1640 containing L-glutamine
B. 2X freezing media
   20% DMSO + 20% FBS + 60% RPMI

1. While *gently* swirling the tube, add drop-wise enough at 4°C 2X freezing medium to

Avoid any further mixing or agitation of the cells.

2. Slowly remove the cell suspension and dispense 1ml per cryovial
3. Place the cryovials in a pre-cooled StrataCooler™ that has been filled with 70% isopropanol according to the manufacturer's instructions.
4. Place the freezing container at –80oC.

5. After 1-3 days at –80 ˚C, vials should be transferred from StrataCooler™ units into standard freezer boxes
6. Transfer the box into a liquid N2 container if necessary

Protocol 2: PBMCs thawing

Thawing Media: RPMI 1440 (Complete RPMI with HI FBS with 200 IU Penicillin, 200 µg/mL Streptomycin), and 2 mM L-Glutamine)

1. Removing cryovials from LN2 Freezer
    - Place immediately on dry Ice or Mr. Frosty equilibrated to -80°C
2. Transfer the cryovial into the 37°C water bath soon.
3. Hold the cryovial in the surface of the water bath with an occasional gentle "flick" during thawing.
4. Do not leave cryovial unattended during the thawing process. (It is important for cell viability that the cells are thawed and processed quickly).
5. When a small bit of ice remains (approximately 1 minute) in the cryovial, transfer the cryovial to the biosafety hood.
6. Dry off the outside of the cryovials with a delicate task wipe before opening to prevent contamination.
7. Add 1mL Thawing Media slowly, in a drop wise manner
8. Mix the cryovials contents by slowly pipetting up/down 3-5 times.
9. Transfer the suspension to 50mL/15mL tube
10. Very slowly add an additional 8 mL of Thawing Media
11. Centrifuge at 400 x g for 10 minutes.
12. Decant the supernatant
13. Gently re-suspend pellet in residual media using by finger flicking the tube
14. Repeat washing once
15. Count the cells and adjust the cells to 10^6/ml
16. Incubate 14 -18 hrs.

Protocol 4: Reagents preparation

A). Protocol for making 4% Paraformaldehyde (100ml)

For 100 ml of a 4% paraformaldehyde stock solution:

Weigh out 4 g of paraformaldehyde, transfer powder into 50 ml conical tube (Falcon).

1. Add 80 ml of dH2O
2. Add 50 µl of 1 M NaOH
3. Heat to 70^0C (cap on), mix frequently until complete solubilization (~10 min the solution should become clear
4. Put on ice and allow the solution to cool down to ~ room temperature
5. Adjust volume to 90 ml with ddH2O
6. Add 1 ml of PBS 10X and mix.
7. Filter and store at 4^0C.

B). Protocol penicillin solution preparation

Available reagent: Penicillin G sodium salt powder

To make 100mL solution of penicillin (100,000 units/L)

Solubility: H_2O: soluble100 mg/mL

1. Prepare 100mL dH_2O
2. Weigh 100mg Penicillin G sodium salt powder and add to Prepare 100mL dH_2O
3. filter sterilize
4. store at -20°C for extended periods or at 2-8°C for 1 week

Recommended for use in cell culture media at 100,000 units/L

C). Protocol for preparation of Streptomycin solution

Available as streptomycin Sulfate salt

To make 100mL solution of Streptomycin

1. Prepare 100mL dH_2O
2. Weigh 100mg Streptomycin salt powder and add to Prepare 1000mL dH_2O
3. filter sterilize
4. store at -20°C for extended periods or at 2-8°C for 1 one month

Recommended for use in cell culture applications at 100 mg/L

D). **FACS buffer**

FACS buffer (1x): contains 1X PBS + 1% FCS + 0.02% sodium azide

□□□□ **Preparation (500mL)**:□□485ml 1XPBS + 5ml FBS + 10ml of 1% sodium azide

E). 10**% AB serum**

10% AB serum (100mL) = 10mL AB serum + 90mL PBS

Protocol 5: FACS staining

Extracellular and intracellular flow cytometry staining

1. Collect cells (0.2- 1x10^6/ml) and transfer into the FACS tubes
2. Spin at 400g (1400 rpm) for 5 minutes at RT: consider the centrifuge R if rpm used
3. Discard the supernatant
4. Resuspend in 1mL PBS
5. Spin as above
6. Discard the supernatant
7. Resuspend in lmL AB serum10% in PBS (Block non-specific Fc-mediated interactions)
8. Leave in the dark for 10 minutes at 2-8 ^{0}C or RT (Cover with aluminium foil)

In the meantime prepare beads for compensation controls. Compensation controls need extracellular single staining only

9. Spin as above
10. Discard the supernatant
11. Resuspend cells in lit bit buffer (approximately 50µl)
12. Add your extracellular antibodies (CD3,CD4,CD8,CD25 CD127) to the cells

NB: the total volume shouldn't be greater than 100 µl

A. Main panel for all samples

CD3 V450	CD4 AH 780	CD8 PerCy5.5	CD25 PECy7	CD127 APC	CD161 PE
2.5µl	4.0µl	1.5µl	1.0µl	2.5µl	2.5µl

+ FoxP3 + live/dead

B. Used for 10 ENL and 10LL from each time point (1st , 2nd and 3rd round)

CD3 V450	CD4 AH 780	CD8 PerCy5.5	CD25 PECy7	CD62L APC	CD45RA PE

2.5µl	4.0µl	1.5µl	1.0µl	10 µl	10 µl	+ FoxP3 + live/dead

C. Optional may be used if required (10 samples same as B)

CD3 V450	CD4 AH 780	CD8 PerCy5.5	CD25 PeCy7	CD62L APC	CD45RO PE	+ FoxP3 + live/dead
2.5µl	4.0µl	1.5µl	1.0µl	20	2.5µl	

13. Add live/ dead satin: to the final concentration of 1µl/mL)
14. Incubate in dark at 4°C for 30 minutes
15. Add 1mL FACS buffer
16. Spin as above and discard the supernatant
17. Re-suspend cells in lit bit buffer

Procedure 18-22 is only for surface staining. Go to number 23 for intracellular staining)

18. Add 200µl PFA (fixing the cells)
19. Fix by keeping in the dark at 4°C for 15 minutes
20. Add 1mL FACS buffer
21. Spin as above and discard the supernatant
22. Add 400µl FACS buffer and read
23. Add 1 mL of Foxp3 Fixation/Permeabilization working solution to each tube and pulse vortex
24. Incubate for 60 minutes at 4^0c in the dark.
25. Without washing , add 2mL of 1X permeablization buffer to each tube
26. Spin as above (400g, 5 minutes , room T) and discard the supernatant
27. Re-suspend pellets in 50 µl 1X permeablization buffer ($FoxP_3$ permeablization wash)
28. Add your intracellular antibody ($FoxP_3$) to the cells: final max. volume =100µl

(To each tube add 10µl anti-FoxP3 antibody)

29. Incubate in dark at 4°C for 30 minutes
30. Without washing, add 2mL 1X permeablization buffer
31. Spin as above and discard the supernatant
32. Add 2mL 1x permeablization buffer

33. Spin as above and discard the supernatant
34. Re-suspend cells in little bit buffer
35. Add 400µl FACS buffer
36. Read

Antibodies volumes/test (for 100 µl total volume)

CD3: 2.5µl

CD4: 5µl
CD8: 1.25µl
CD25: 0.625µl
CD127: 2.5µl
CD161: 2.5µl
FoxP3: 10µl
Live/dead satin: 100µl/ test

For 10 reactions: working example

CD3, CD127, CD161 = each 25µl each in small test tube (Eppendorf) separately
CD4= 50µl
CD8= 12.5µl
CD25= 6.25µl
FoxP3= 100 µl
Live/dead= 1000µl
Notice:
It is possible to add for example 25µl of CD3 and 25µl FACS buffer. But when antibodies are added to each test tube, the volume added must be doubled. Instead of 2.5 it has to be 5 µl

Protocol 6. RNA Extraction

A). Extraction of RNA from skin punch biopsy collected from leprosy patients

To be read in conjunction with the Category 3 code of practice

Organism	*M.leprae* There may be small numbers of live organisms in tissue f from untreated patients
Laboratory	427A
Project supervisor	Prof. Diana Lockwood
Routes of infection	Predominately by aerosol route
Disinfectants	Surfanios, freshly prepared (5%, final concentration), disinfection overnight
Written by	Edessa Negera Gobena April, 2015
Approved by principal investigator: Diana Lockwood	Date: Signature:
Approved copy sent to the safety office:	Date:

Extraction of RNA from skin biopsy collected from leprosy patients

To be used with the RNeasy Fibrous Tissue Mini Kit

General precautions: Apply RNase Away spray to all surfaces (work surface, tubes, pipettes, microcentrifuge and gloves) and wipe down.

In CAT2 lab:

Make DNase solution according to kit guidelines, aliquot and store at -20C.

Before using wash buffer 2 (BR4) add 4 volumes of ethanol (96-100%, purity grade p.a.) to obtain a working solution.

In CAT3 lab:

Wear gown, gloves and sleeves. Risk of aerosol formation through use of pipettes and centrifugation.

TAKE the following into class I hood:

Large 3 polystyrene container full of ice

Microcentrifuge

a. Heating block
b. Mini vortex
c. Aerosol resistant tips and Gilson pipettes will be kept inside a plastic container in the isolator and transferred into the cabinet as required. These must be surface fumigated should they need to be removed from the cabinet.
d. RNeasy Fibrous Tissue Mini Kit
    - RNase-free water (4ml/sample)
    - Secondary BD Hemograd closures
    - Suspension buffer (BR1) (350ul/sample)
    - Binding buffer (BR2) (300ul/sample)
    - Proteinase K (40ul/sample)
    - 100% cold ethanol (350ul/sample)
    - Wash buffer 1 (BR3) (700ul/sample)
    - DNase I (10ul/sample) /DNA digestion buffer (70ul/sample)
    - Wash buffer 2 (1000ul/sample)

- Elution buffer (80ul/sample)
- Purification columns and collection tubes

CAT. III PROCEDURE
PROTOCOL & RISK ASSESSMENT

RNA extraction from tissues collected from leprosy patients (ENL and LL)

1. Tissue samples in RNAlater will be shipped to category 3 lab at LSHTM from Ethiopia (optional).
2. The tissue samples are contained in cryo-tubes and sealed with sealer. The cryo-tubes are kept in cryo-box.
3. Wipe tubes with 5% surftanios. Tissue samples stabilized in RNAlater removed with forceps and placed on suitably sized vessel containing liquid nitrogen and grind thoroughly with a mortar and pestle.
   Notice: any formed crystals should be removed.
4. Transfer tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied with the kit). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw. Add the appropriate volume of Buffer RLT. Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Discard the tissue nunc tubes into % surftanios.
5. Add 590µl RNase-free water to the lysate. Then add 10µl proteinase K solution, and mix thoroughly by pipetting.
6. Incubate at 55°C for 10 min.
7. Centrifuge at 20–25°C for 3 min at 10,000 x g.
   A small pellet of tissue debris will form, sometimes accompanied by a thin layer or film on top of the supernatant.
8. Pipet the supernatant (approximately 900µl) into a new 1.5 ml or 2 ml microcentrifuge tube (not supplied). Discard the pellet into % surftanios.

Transferring any of the pellet. If this is unavoidable, a small amount of pelleted debris may be carried over without affecting the RNeasy procedure. Hold the pipet tip under the thin layer or film on top of the supernatant, if present. This

layer will usually adhere to the outside of the pipet tip and should not be transferred.

9. Add 0.5 volumes (usually 450µl) of ethanol (96–100%) to the cleared lysate. Mix well by pipetting. Do not centrifuge. Precipitates may be visible after addition of ethanol. This does not affect the procedure.
10. Transfer 700µl of the sample, including any precipitate that may have formed, to an RNeasy Mini spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge at 20–25°C for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow through 5% surfatanios.

    Reuse the collection tube in step 11.
11. Repeat step 10 using the remainder of the sample. Discard the flow-through into % surftanios

    Reuse the collection tube in step 12.
12. Add 350µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 15 s at ≥8000 x g (≥10,000 rpm) to wash the membrane. Discard the flow through 5% surfatanios.

    Reuse the collection tube in step 15.

    Optional: If on-column DNase digestion is not desired, add 700µl Buffer RW1 instead, centrifuge for 15 s at ≥8000 x g, and discard the flow-through* (but not the collection tube). Proceed to step 16.

13. Add 10µl DNase I stock solution to 70µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube. Note: **DNase** I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.

14. Add the DNase I incubation mix (80µl) directly to the RNeasy spin column membrane, and place on the benchtop (20–30°C) for 15 min. Note: Be sure to add the DNase I incubation mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

15. Add 350µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at _8000 x g (_10,000 rpm) at 20–25°C. Discard the flow through into % surftanios

    Reuse the collection tube in step 16.

16. Add 500µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 15 s at ≥8000 x g (≥10,000 rpm) to wash the membrane. Discard the flow-through into 5% suratanios

    Reuse the collection tube in step 17

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting").

17. 15Add 500µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 2 min at ≥8000 x g (≥10,000 rpm) to wash the membrane.

    The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

    Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

18. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 17.
19. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50µl Nase-free water directly to the RNeasy spin column membrane. Close the lid gently. To elute the RNA, centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) at 20–25°C.
20. Repeat step 19 using another 30–50µl RNase-free water, or using the eluate from step 19 (if high RNA concentration is required). Reuse the collection

tube from step 19. If the expected RNA yield is >30µg, there is no need to repeat step 19.

If using the elute from step 19, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

21. RNA samples to be stored in a named container at -80 ^{0}C.
    Wipe tubes and box with 5% surfanios.

22. Clear up.
    Waste to be left overnight in a waste box with lid on containing final concentration of 5% surfanios. Next day waste to be put in autoclave boxes and taken to CSU in closed metal trolley. Hood to be swabbed down with 5% surfanios or bombed as appropriate.

23. Fill in log book
24. Proceed to cDNA synthesis

SUMMARY PROTOCOL & RISK ASSESSMENT

B). Extraction of RNA from blood collected from leprosy patients

To be read in conjunction with the Category 3 code of practice

Organism	*M.leprae* There may be small numbers of live organisms in blood from untreated patients
Laboratory	427A
Project supervisor	Diana Lockwood
Routes of infection	Predominately by aerosol route
Disinfectants	Surfanios, freshly prepared (5%, final concentration), disinfection overnight
Written by	Edessa N Gobena May, 2015
Approved by principal investigator: Diana Lockwood	Date: Signature:
Approved copy sent to the safety office:	Date:

B). Extraction of RNA from blood collected from leprosy patients

To be used with the PAXgene Blood RNA kit.

General precautions: Apply RNase Away spray to all surfaces (work surface, tubes, pipettes, microcentrifuge and gloves) and wipe down.

In CAT2 lab:

Make DNase solution according to kit guidelines, aliquot and store at -20C.

Before using wash buffer 2 (BR4) add 4 volumes of ethanol (96-100%, purity grade p.a.) to obtain a working solution.

In CAT3 lab:

Wear gown, gloves and sleeves. Risk of aerosol formation through use of pipettes and centrifugation.

TAKE the following into class I hood:

e. Large3 polystyrene container full of ice
f. Microcentrifuge
g. Heating block
h. Mini vortex
i. Aerosol resistant tips and Gilson pipettes will be kept inside a plastic container in the isolator and transferred into the cabinet as required. These must be surface fumigated should they need to be removed from the cabinet.
j. PAXgene Blood RNA kit items:
    - RNase-free water (4ml/sample)
    - Secondary BD Hemograd closures
    - Suspension buffer (BR1) (350ul/sample)
    - Binding buffer (BR2) (300ul/sample)
    - Proteinase K (40ul/sample)
    - 100% cold ethanol (350ul/sample)
    - Wash buffer 1 (BR3) (700ul/sample)
    - DNase I (10ul/sample) /DNA digestion buffer (70ul/sample)
    - Wash buffer 2 (1000ul/sample)

- Elution buffer (80ul/sample)
- Purification columns and collection tubes

CAT. III PROCEDURE PROTOCOL & RISK ASSESSMENT

RNA extraction from blood collected from leprosy patients (ENL)

25. Blood transported from hospital to Cat. 3 at LSHTM.
    Blood will be contained in a sealed unbreakable primary container and held within a secondary approved canister with sufficient absorbent material between the two to absorb any spillage according to Post Office transportation guidelines.

26. In Cat. 3 room blood will be removed from canister in class 2 hood.
    Double glove procedure to be used in the first 4 stages and thereafter single gloves. Class 2 hood will contain tray containing absorbent material and 5% fresh surfanios (final concentration) from beginning to end of experimental protocol.

27. PAxgene tubes containing blood will be left overnight at RT before starting the procedure to ensure complete lysis of blood cells.
28. Blood centrifuged at 3000-5000g for 10 minutes at room temperature.
    Wipe tubes with 5% surftanios. Open centrifuge buckets in hood and put in tubes carefully and replace bucket lids. Wipe buckets before removing from class 2 hood and placing in the centrifuge.

29. Using pastette remove supernatant and add 4ml RNase free water to the pellet and close the tube using a fresh secondary BD Hemogard closure (supplied with the kit) and vortex until the pellet is visibly dissolved.
30. Centrifuge at 3000-5000g for 10 minutes at room temperature.
    Reduce aerosol by keeping tip below surface of the water. Waste into 5% surfanios.

31. Using pastette remove entire supernatant and add 350ul resuspension buffer (BR1) and vortex until pellet is visibly dissolved. Waste into 5% surfanios.

32. Pipet sample to a 1.5ml microcentrifuge tube and add 300ul binding buffer (BR2) and 40ul proteinase K (PK). Mix by vortexing for 5 sec and incubate at 55 C (heating block).
    Waste into 5% surfanios. Wipe microcentrifuge tubes with 5% surfanios and continue protocol outside the hood on clean benchtop in tray containing absorbent paper. Freshly prepared 5% surfanios waste bucket on bench top.
33. Pipet the lysate directly into a PAXgene Shredder spin column (lilac) placed in a 2ml processing tube.
34. Centrifuge for 3 mins at max speed in microcentrifuge (not to exceed 20,000g).
    Waste into 5% surfanios.
35. Transfer the entire supernatant of the flow-through fraction to a fresh 1.5ml microcentrifuge tube without disturbing the pellet in the processing tube.
36. Add 300ul ethanol 96-100%, mix by vortex and centrifuge briefly (1-2sec at 500-1000g).
    Waste into 5% surfanios.
37. Apply 700ul sample to PAXgene spin column sitting in a 2ml processing tube and discard the old processing tube containing flow-through.
38. Pipet 350ul wash buffer (BR3) into the PAXgene RNA spin column.
39. Centrifuge for 1 min at 8000g discard flow through.
    Waste into 5% surfanios.
40. Add 10ul DNAse I stock solution to 70ul DNA digestion buffer in a 1.5ml microcentrifuge tube. Mix gently by flicking the tube.
41. Apply 10ul Dnase1 and 70ul DNA digestion buiffer (RDD) to PAXgene RNA spin column membrane and leave on bench top for 15 mins.
42. Pipet 350ul wash buffer (BR3) into the PAXgene RNA spin column ande centrifuge for 1min at 8000g. Place the spin column in a new 2ml processing tube and discard the old processing tube containing flow-through in 5% surfanios.
43. Pipet 500ul washing buffer 2 (BR4) to column and centrifuge for 1 min at 8000g discard flow through.

Waste into 5% surfanios.

44. Repeat step 18.
45. Discard tube containing flow through and place the column in a new 2ml processing tube. Centrifuge for 1 in at 8000g
46. Discard the processing tube containing the flow-through in 5%surfanios.
47. Place the column in a 1.5ml microcentrifuge tube and pipet 40ul elution buffer (BR5) directly onto column membrane.
48. Centrifuge for 1min at 8000g to elute the RNA.
49. Repeat the step23 using 40ul elution buffer and the same microcentrifuge tube.
50. Incubate elute at 65°C for 5 min. and chill immediately on ice.
    Discard ice into 5% surfanios.
51. RNA samples to be stored in a named container at -80 ^{0}C.
    Wipe tubes and box with 5% surfanios.
52. Clear up.
    Waste to be left overnight in a waste box with lid on containing final concentration of 5% surfanios. Next day waste to be put in autoclave boxes and taken to CSU in closed metal trolley. Hood to be swabbed down with 5% surfanios or bombed as appropriate.
53. Fill in log book
54. Continue cDNA synthesis

Protocol 7: cDNA synthesis for real-time two-step RT-PCR

1. Allow the components to thaw on ice
2. Referring to the table below, calculate the volume of components needed to prepare **the required number of reactions.**

Note: Prepare the RT master mix on ice.

Component	Volume / reaction (µl)
10× RT Buffer	2.0
25× dNTP Mix (100 mM)	0.8
10× RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H2O	3. 2
Total per Reaction	10.0

3. Place the 2× RT master mix on ice and mix gently.

To prepare the cDNA RT reactions:

1. Pipette 10 µL of 2× RT master mix into individual tube.
2. Pipette 10 µL of RNA sample into tube, pipetting up and down two times to mix
3. Seal the tubes
4. Briefly centrifuge the tubes to spin down the contents and to eliminate any air bubbles.
5. Place the plate or tubes on ice until you are ready to load the thermal cycler.

To perform reverse transcription:

1. Program the thermal cycler conditions

MPORTANT! These conditions are optimized for use with the High Capacity cDNA Reverse Transcription Kits (by the company).

Condition	Step 1	Step 2	Step 3	Step 4
Temp. (^{0}C)	25	37	85	4
Time	10 min	120 min	5 min	∞

2. Set the reaction volume to 20 µL.
3. Load the reactions into the thermal cycler
4. Start the reverse transcription run

cDNA storage

Storage Duration Storage	Temperature (°C)
Short-term (up to 24 hours before use)	2-6
Long term	−15 to −25

Protocol 8: Real-Time PCR

PCR machine: Rotor-Gene 3000

Important points before starting

1. The Rotor-Gene SYBR Green PCR Kit has been developed for use in a two-step cycling protocol, with a denaturation step at 95°C and a combined annealing/extension step at 60°C. This protocol will also work for primers with a *T*m well below 60°C.
2. For the highest efficiency in real-time PCR using SYBR Green I, targets should ideally be 60–200bp in length.
3. The PCR must start with an initial incubation step of 5 minutes at 95°C to activate HotStarTaq *Plus* DNA Polymerase.
4. We recommend a final reaction volume of 25 µl.
5. Always start with the Mg2+ concentration as provided in 2x Rotor-Gene SYBR Green PCR Master Mix.

Procedure

1. Thaw 2x Rotor-Gene SYBR Green PCR Master Mix, template DNA or cDNA, primers, and RNase-free water. Mix the individual solutions.
2. Prepare a reaction mix according to the following Table. Due to the hot start, it is not necessary to keep samples on ice during reaction setup or while programming the Rotor-Gene cycler.

Note: We strongly recommend starting with the Mg2+ concentration as provided in 2x Rotor-Gene SYBR Green PCR Master Mix.

Component	Volume/reaction (µl)	Final concentration
2x Rotor-Gene SYBR Green PCR Master Mix	6.25	1x
Primer FW	1.0	1µm
Primer R	1.0	1µm
RNase-free water	3.25	
Template DNA or cDNA (added at step 4)	1.0	≤100 ng/ reaction
Total reaction volume l	12	

3. Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes.
4. Add template DNA or cDNA (≤100 ng/reaction) to the individual PCR tubes containing the reaction mix.

For two-step RT-PCR, the volume of the cDNA added (from the undiluted RT reaction) should not exceed 10% of the final PCR volume.

5. Program the Rotor-Gene cycler according to the program outlined in the following table

   Data acquisition should be performed during the combined annealing/extension step

Step	Time	Temperature
PCR initial activation step	15 min	95^0C
Number of cycles	40	
Denaturation	5s	95^0C
annealing	10s	60^0C
extension	20s	72^0C

6. Place the PCR tubes in the Rotor-Gene cycler, and start the cycling program.

7. Optional: Perform melting curve analysis of the PCR product(s) to verify their specificity and identity (50ºC to 99ºC increasing by 1ºC on each step. Use the interval between increases in temperature 30 seconds for the first step and then 5 seconds for subsequent steps

Primers

Primer (F, forward; R, reverse)	Sequence (5' to 3')	Length	T_m (ºC)	% GC ratio	Amplicon size (bp)	Accession number
IL-10 FW	TGAGAACCAAGACCCAGACA	20	63.4	50	182	
IL-10 RV	TCATGGCTTTGTAGATGCCT	20	62.2	45		NM_000594
TNF-a FW	AGCCCATGTTGTAGCAAACC	20	58.74	50.00	104	
TNF-a RV	GCTGGTTATCTCTCAGCTCCA	21	59.24	52.38		NM_172369
IL-17A FW	AGACCTCATTGGTGTCACTGC	21	64.2	52.3	238	
IL-17A RV	CTCTCAGGGTCCTCATTGCG	20	67.2	60		NM_002190
IL-6 FW	TTCGGTCCAGTTGCCTTCTC	20	66.5	55	193	
IL-6 RV	TACATGTCTCCTTTCTCAGGGC	22	64.3	50		NM_000600
IL-1B FW	AGCCCCAGCCAACTCAATTC	20	67.4	55	380	
IL-1B RV	CATGGAGAACACCACTTGTTGC	22	66.6	50		XM_006712496
Fox P3 FW	TGGAGAAGGAGAAGCTGAGTGC	22	61.99	54.55	73	
FoxP3 RV	ACAGATGAAGCCTTGGTCAGTGC	23	63.24	52.17		XM_011543919
IFN-g FW	GGCTTTTCAGCTCTGCATCG	20	59.90	55.0	172	
INF-g RV	TCTGTCACTCTCCTCTTTCCA	21	57.76	47.62		NM_000619
IL-8 FW	ACCGGAAGGAACCATCTCAC	20	59.39	55.0	104	
IL-8 RV	AAACTGCACCTTCACACAGAG	21	58.71	47.62		NM_000584
HuPO FW *	GCTTCCTGGAGGGTGTCC	18	59.33	66.67	105	
HuPO RV *	GGACTCGTTTGTACCCGTTG	20	58.86	55.00		NM_001002
TGF-β FW	ACATCAACGCAGGGTTCACT	20	59.89	50	264	XM_011527242
TGF-β RV	GAAGTTGGCATGGTAGCCC	20	60.03	55		
C1-qA FW	ATGGTGACCGAGGACTTGTG	20	59.68	55	276	NM_015991
C1-qA RV	GTCCTTGATGTTTCCTGGGC	20	58.82	55		
C1-qB FW	CAGGTTGAAATCAGCATTGCC	21	58.39	47.62	163	XM_011542059
C1-qB RV	CTGTGTCAGACGCCTCCTTTC	21	60.94	57.14		
C1-qC FW	AAGGATGGGTACGACGGACTG	21	61.56	57.14	213	NM_172369
C1-qC RV	TTTCTGCTTGTATCTGCCCTC	21	58.01	47.62		

*Housekeeping gene

Appendix 5: Tables for regulatory T-cell results

Table 1. Median percentage of CD4+T-cells, CD8+ T-cells and the median percentage ratio of CD4+ to CD8+ T- cells in the PBMCs of patients with ENL reaction and LL patient controls before during and after treatment of ENL cases with prednisolone.

time	cell type	Patients	Median (%)	P	U	H-L	95% Confidence interval
before LL=31 ENL=46	CD4+	ENL	61.30	< 0.0001	153.5	-12.80	-16.20 to -9.200
		LL	49.10				
	CD8+	ENL	27.00	< 0.0001	275	8.200	4.900 to 12.00
		LL	35.70				
	CD4/CD8	ENL	2.25	< 0.0001	174	-0.9000	-1.200 to -0.6000
		LL	1.4				
during LL=31 ENL=46	CD4+	ENL	54.20	0.0235	368.5	3.800	0.5000 to 8.600
		LL	61.35				
	CD8+	ENL	34.4	0.0001	250	-7.300	-10.80 to -3.400
		LL	28.3				
	CD4/CD8	ENL	1.7	0.001	293	0.6000	0.2000 to 1.000
		LL	2.25				
after LL=28 ENL=44	CD4+	ENL	58.10	0.0033	364.5	8.050	2.600 to 13.30
		LL	64.70				
	CD8+	ENL	33.35	0.0313	430	-5.400	-10.00 to -0.5000
		LL	27.2				
	CD4/CD8	ENL	2.14	0.0944	471	0.3700	-0.0500 to 0.8200
		LL	1.8				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 2. Median percentage of CD25 expression on CD4+ and CD8+ T-cells in the PBMCs of patients with ENL reaction and LL patient controls before during and after treatment of ENL cases with prednisolone

time	cell type	Patients	Median (%)	P	U	H-L	95% Confidence interval
before LL=31 ENL=46	CD4+CD25+	ENL	**8.920**	0.8143	690.0	0.1900	-1.290 to 1.570
		LL	8.750				
	CD8+CD25+	ENL	2.580	0.0465	521.5	0.6800	0.01000 to 1.320
		LL	3.320				
during LL=31 ENL=46	CD4+CD25+	ENL	8.170	0.8970	535.5	-0.08500	-1.860 to 1.660
		LL	7.140				
	CD8+CD25+	ENL	3.050	0.1582	434.5	0.4850	-0.2200 to 1.700
		LL	3.170				
after LL=28 ENL=44	CD4+CD25+	ENL	9.455	0.0840	484.5	-1.295	-2.660 to 0.1500
		LL	7.580				
	CD8+CD25+	ENL	2.245	0.0858	467.0	0.4500	-0.1000 to 1.110

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 3. Median percentage of FoxP3 expression on CD4+ and CD8+ T-cells in the PBMCs of patients with ENL reaction and LL patient controls before during and after treatment of ENL cases with prednisolone.

time	cell type	Patients	Median (%)	P	U	LH	95% confidence interval
before LL=31 ENL=46	CD4+FoxP3+	ENL	2.105	< 0.0001	230.5	2.380	1.540 to 3.260
		LL	5.13				
	CD8+FoxP3+	ENL	0.585	0.0701	538.5	0.1800	-0.0100 to 0.380
		LL	0.71				
during LL=31 ENL=46	CD4+FoxP3+	ENL	3.52	0.016	357.5	-1.160	-2.020 to -0.290
		LL	2.595				
	CD8+FoxP3+	ENL	0.49	0.0108	347	0.4850	-0.2200 to 1.700
		LL	1.165				
after LL=28 ENL=44	CD4+FoxP3+	ENL	4.37	0.0537	449	-0.5950	-1.340 to 0.0300
		LL	3.79				
	CD8+FoxP3+	ENL	0.5	0.4817	554.5	0.0700	-0.1200 to 0.2800
		LL	0.705				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 4. Median percentage of CD4 and CD8 T-cells expressing CD25 and FoxP3 in the PBMCs of patients with ENL reaction and LL patient controls before during and after treatment of ENL cases with prednisolone.

time	cell type	Patients	Median (%)	P	U	LH	95 % Confidence interval
before LL=31 ENL=46	CD4+CD25+FoxP3+	ENL	1.785	< 0.0001	189.5	1.995	1.430 to 2.470
		LL	3.800				
	CD8+CD25+FoxP3+	ENL	0.4150	0.1333	568.0	0.1500	-0.0600 to 0.410
		LL	0.5900				
during LL=31 ENL=46	CD4+CD25+FoxP3+	ENL	2.620	0.0755	406.0	-0.6300	-1.300 to 0.09000
		LL	2.450				
	CD8+CD25+FoxP3+	ENL	0.2800	0.3708	475.0	0.1050	-0.0880 to 0.3800
		LL	0.5150				
after LL=28 ENL=44	CD4+CD25+FoxP3+	ENL	3.295	0.0003	312.5	-1.075	-1.680 to -0.5100
		LL	2.220				
	CD8+CD25+FoxP3+	ENL	0.3450	0.0969	472.0	0.1500	-0.0210 to 0.4200
		LL	0.5750				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 5. Median percentage of CD4+ and CD8+ regulatory T- cells in the PBMCs of patients with ENL reaction and LL patient controls before, during and after treatment of ENL cases with prednisolone.

time	cell type	Patients	Median (%)	P	U	H-L	95% confidence interval
before LL=31 ENL=46	CD4+Tregs	ENL	1.67	< 0.0001	182.0	1.925	1.390 to 2.380
		LL	3.79				
	CD8+Tregs	ENL	0.3665	0.3923	630.0	0.0750	-0.1310 to 0.2610
		LL	0.5400				
during LL=31 ENL=46	CD4+Tregs	ENL	3.21	0.0745	405.5	-0.5700	-1.190 to 0.08000
		LL	2.43				
	CD8+Tregs	ENL	0.2340	0.0879	411.5	0.1270	-0.0120 to 0.3490
		LL	0.4150				
after LL=28 ENL=44	CD4+Tregs	ENL	3.21	0.0090	391.5	-0.7300	-1.290 to -0.2200
		LL	2.480				
	CD8+Tregs	ENL	0.3425	0.2191	509.0	0.0980	-0.0620 to 0.3190
		LL	0.4745				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 6. Median percentage of CD4 and CD8 T-cells expressing CD161 (IL-17 producing TH17) in the PBMCs of patients with ENL reaction and LL patient controls before during and after treatment of ENL cases with prednisolone

time	cell type	Patients	Median (%)	P	U	H-L	95% confidence interval
before LL=31 ENL=46	CD4+CD161+	ENL	20.35	0.0122	473.0	-4.700	-8.100 to -1.000
		LL	15.10				
	CD8+CD161+	ENL	7.120	0.2807	608.5	1.005	-1.110 to 2.930
		LL	7.900				
during LL=31 ENL=46	CD4+CD161+	ENL	14.40	0.2891	462.0	1.320	-0.8000 to 3.700
		LL	16.70				
	CD8+CD161+	ENL	6.140	0.2376	452.5	1.140	-0.9200 to 3.250
		LL	6.350				
after LL=28 ENL=44	CD4+CD161+	ENL	19.00	0.8701	601.5	0.4000	-3.300 to 4.000
		LL	19.15				
	CD8+CD161+	ENL	7.380	0.6358	574.5	0.4750	-1.700 to 2.760
		LL	9.290				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 7. Median Percentage of CD4+T-cells, CD8+ T-cells and the median percentage ratio of CD4 to CD8+ T- cells in the PBMCs of patients with ENL reactions before during and after treatment with prednisolone.

Cell type	Patients	Median (%)	P	H-L	U	95% Confidence interval for HL
CD4+	1st	61.3	0.0006	6.800	515	-10.40 to -2.900
	2nd	54.2				
CD4+	1st	61.3	0.0234	-4.600	768.5	-8.300 to -0.4000
	3rd	57.25				
CD4+	2nd	54.2	0.3483	2.200	790.0	-2.100 to 6.800
	3rd	57.25				
CD8+	1st	27.00	< 0.0001	6.800	432.0	3.700 to 10.10
	2nd	34.40				
CD8+	1st	27.00	0.0028	6.000	678.5	2.000 to 9.800
	3rd	34.30				
CD8+	2nd	34.40	840.5	-1.000	840.5	-4.800 to 2.700
	3rd	34.30				
CD4+/CD8+	1st	2.250	< 0.0001	-0.6000	439.5	-0.900 to -0.300
	2nd	1.700				
CD4+/CD8+	1st	2.250	0.0056	-0.5000	706.0	-0.800 to -0.100
	3rd	1.800				
CD4+/CD8+	2nd	1.700	0.3339	0.1000	787.0	-0.100 to 0.400
	3rd	1.800				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

ENL 1st, 2nd, 3rd (n= 46, 46, 44 for 1st, 2nd and 3rd rounds respectively)

Table 8. Median percentage of CD4 and CD8 T-cells expressing CD25 and FoxP3 in the PBMCs of patients with ENL reaction before during and after treatment with prednisolone.

Cell type	Patients	Median (%)	P	HL	U	95% Confidence interval for HL
CD4+CD25+	1st	8.920	0.4436	-0.4600	809.5	-1.950 to 0.8100
	2nd	8.170				
CD4+CD25+	1st	8.920	0.4881	0.4250	925.5	-0.9100 to 1.620
	3rd	9.455				
CD4+CD25+	2nd	8.170	0.1507	1.000	700.0	-0.3400 to 2.260
	3rd	9.455				
CD8+CD25+	1st	2.580	0.8043	0.1300	868.5	-0.7800 to 0.9400
	2nd	3.050				
CD8+CD25+	1st	2.580	0.5009	-0.1950	928.0	-0.7600 to 0.4300
	3rd	2.245				
CD8+CD25+	2nd	3.050	0.6385	-0.2700	806.0	-1.070 to 0.9400
	3rd	2.245				
CD4+FoxP3+	1st	2.105	< 0.0001	1.405	410.5	0.8500 to 2.000
	2nd	3.520				
CD4+FoxP3+	1st	2.105	< 0.0001	2.155	297.0	1.600 to 2.740
	3rd	4.370				
CD4+FoxP3+	2nd	3.520	0.0352	0.8250	627.5	0.07000 to 1.350
	3rd	4.370				
CD8+FoxP3+	1st	0.5850	0.3667	0.09700	794.0	-0.1200 to 0.3200
	2nd	0.4900				
CD8+FoxP3+	1st	0.5850	0.8866	0.01000	994.0	-0.1500 to 0.1700
	3rd	0.5000				
CD8+FoxP3+	2nd	0.4900	0.4096	-0.07000	767.0	-0.2600 to 0.1000
	3rd	0.5000				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 9. Median percentage of CD25+FoxP3+ expression on CD4 and CD8 T-cells in the PBMCs of patients with ENL reaction before during and after treatment with prednisolone.

Cell type	Patients	Median (%)	P	HL	U	95% Confidence interval for HL
CD4+CD25+FoxP3+	1st	1.785	< 0.0001	1.040	397.5	0.6300 to 1.650
	2nd	2.620				
CD4+CD25+FoxP3+	1st	1.785	< 0.0001	1.550	352.0	1.080 to 2.000
	3rd	3.295				
CD4+CD25+FoxP3+	2nd	2.620	0.1846	0.4500	712.0	-0.2500 to 0.9100
	3rd	3.295				
CD8+CD25+FoxP3+	1st	0.4150	0.6308	-0.04000		-0.2400 to 0.09900
	2nd	0.2800				
CD8+CD25+FoxP3+	1st	0.4150	0.4931	-0.0600	926.5	-0.2700 to 0.0900
	3rd	0.3450				
CD8+CD25+FoxP3+	2nd	0.2800	0.9042	0.01000	844.5	-0.1100 to 0.1200
	3rd	0.3450				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 10: Median percentage of CD4+ and CD8+ Tregs in the PBMCs of patients with ENL reaction before during and after treatment with prednisolone. B) Trends in frequency of individual $CD4^+$ and $CD8^+$ Tregs in the PBMCs of patients with ENL reaction before and after treatment with prednisolone.

Cell type	Patients	Median (%)	P	HL	U	95% Confidence interval for HL
CD4+Tregs	1st	1.670	< 0.0001	1.010	348.0	0.6200 to 1.480
	2nd	2.460				
CD4+Tregs	1st	1.670	< 0.0001	1.600	313.5	1.170 to 2.030
	3rd	3.210				
CD4+Tregs	2nd	2.460	0.1063	0.560	713.5	-0.090 to 0.940
	3rd	3.210				
CD8+Tregs	1st	0.3665	0.0451	-0.1275	670.0	-0.3100 to -0.0010
	2nd	0.2340				
CD8+Tregs	1st	0.3665	0.6904	-0.0275	1007	-0.2100 to 0.1030
	3rd	0.3575				
CD8+Tregs	2nd	0.2340	0.0884	0.1020	703.5	-0.0190 to 0.2090
	3rd	0.3575				

P= probability (significant at $P \leq 0.05$); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 11. IL-17 Producing cells in the PBMCs of patients with ENL and LL controls before, during and after treatment

Time point	cell type	patients	median %	P	U	LH	95 CI
Before treatment	IL-17 producing T cells	ENL	23.10	0.0346	436.5	-4.450	-8.500 to -0.3000
		LL	18.40				
	IL-17 producing lymphocytes	ENL	26.45	0.0059	382.5	-5.700	-9.100 to -1.700
		LL	20.60				
	CD4+ IL-17+	ENL	20.35	0.0122	473.0	-4.700	-8.100 to -1.000
		LL	15.10				
	CD8+IL-17+	ENL	7.120	0.2807	608.5	1.005	-1.110 to 2.930
		LL	7.900				
During treatment	IL-17 producing T cells	ENL	16.40	0.2132	447.5	2.000	-1.500 to 5.200
		LL	18.15				
	IL-17 producing lymphocytes	ENL	15.40	0.0954	414.5	3.200	-0.6000 to 6.500
		LL	19.70				
	CD4+ CD161+	ENL	14.40	0.2891	462.0	1.320	-0.8000 to 3.700
		LL	16.70				
	CD8+CD161+	ENL	6.140	0.2376	452.5	1.140	-0.9200 to 3.250
		LL	6.350				
After treatment	IL-17 producing T cells	ENL	13.25	0.1142	497.5	2.725	-0.6700 to 6.800
		LL	16.30				
	IL-17 producing lymphocytes	ENL	12.70	0.6279	594.5	0.9550	-2.900 to 4.400
		LL	14.80				
	CD4+ CD161+	ENL	19.00	0.8701	601.5	0.4000	-3.300 to 4.000
		LL	19.15				
	CD8+CD161+	ENL	7.380	0.6358	574.5	0.4750	-1.700 to 2.760
		LL	9.290				

Appendix 6: Tables for Memory T-cell results

Table 1. Memory T- cell expression on $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the PBMCs of ENL cases and LL control patients before, during and after treatment.

time	cell type	Patients	Median (%)	P	U	H-L	95 % Confidence interval
Before treatment ENL=35 LL=25	$CD3^+CD45RO^+$	ENL	41.05	0.0010	261.0	-10.50	-17.80 to -4.600
		LL	28.80				
	$CD4^+CD45RO^+$	ENL	52.30	< 0.0001	159.0	-20.30	-27.40 to -11.90
		LL	30.50				
	$CD8^+CD45RO^+$	ENL	17.50	0.3425	429.0	-2.460	-7.900 to 2.400
		LL	15.20				
during treatment ENL=35 LL=25	CD3+CD45RO+	ENL	29.20	0.8957	360.0	-0.4500	-7.700 to 6.400
		LL	43.8				
	CD4+CD45RO+	ENL	45.9	0.9966	367.5	-0.05	-13.90 to 11.20
		LL	43.00				
	CD8+CD45RO+	ENL	12.55	0.8029	353.0	-0.6000	-4.460 to 4.300
		LL	13.00				
After treatment ENL=35 LL=25	CD3+CD45RO+	ENL	31.15	0.4242	458.5	2.200	-3.600 to 8.600
		LL	32.65				
	CD4+CD45RO+	ENL	45.00	0.5207	470.5	-2.500	-10.50 to 6.100
		LL	41.80				
	CD8+CD45RO+	ENL	12.85	0.1897	419.5	3.365	-1.790 to 9.680
		LL	15.15				

P= probability (significant at $P \leq 0.05$); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 2. Median percentage of activated T-cells among $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the PBMCs of ENL cases and LL control patients before, during and after treatment.

Time	cell type	Patients	Median (%)	P	U	HL	95 % Confidence interval
before LL=25 ENL=35	$CD3^+CD62L^-$	ENL	59.15	< 0.0001	55.50	-22.40	-30.00 to -17.10
		LL	37.70				
	$CD4^+CD62L^-$	ENL	50.65	< 0.0001	181.5	-19.10	-27.70 to -11.50
		LL	27.10				
	$CD8^+CD62L^-$	ENL	71.15	< 0.0001	76.00	-27.90	-34.50 to -20.20
		LL	45.40				
during LL=25 ENL=35	CD3+CD62L-	ENL	46.95	0.7833	351.5	3.095	-11.10 to 17.40
		LL	49.20				
	CD4+CD62L-	ENL	29.65	0.5555	318.0	3.350	-6.400 to 12.60
		LL	38.05				
	CD8+CD62L-	ENL	59.50	0.3981	318.0	-5.800	-21.10 to 9.700
		LL	49.70				
LL=25 ENL=35	CD3+CD62L-	ENL	33.90	0.1704	415.0	5.650	-2.800 to 13.60
		LL	41.35				
	CD4+CD62L-	ENL	29.35	0.7079	491.0	1.800	-4.900 to 8.900
		LL	32.95				
	CD8+CD62L-	ENL	34.45	0.0149	335.5	10.10	1.600 to 18.60
		LL	45.25				

P= probability (significant at $P \leq 0.05$); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 3. Median percentage of activated memory T-cells expressed by $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the PBMCs of ENL cases and LL control patients before, during and after treatment

time	cell type	Patients	Median (%)	P	U	H-L	95 % Confidence interval
before LL=25 ENL=35	$CD3^+EM$	ENL	26.55	< 0.0001	100.5	-18.29	-22.80 to -12.80
		LL	7.950				
	$CD4^+EM$	ENL	24.55	< 0.0001	68.00	-18.40	-23.05 to -13.50
		LL	8.850				
	$CD8^+EM$	ENL	16.50	0.0083	306.0	-6.690	-11.45 to -2.200
		LL	7.200				
during LL=25 ENL=35	$CD3^+EM$	ENL	16.80	0.4483	323.0	-2.415	-7.530 to 2.810
		LL	13.00				
	$CD4^+EM$	ENL	11.40	0.0284	240.0	5.825	0.6900 to 11.70
		LL	17.60				
	$CD8^+EM$	ENL	7.530	0.8690	358.0	0.2800	-2.850 to 3.410
		LL	8.340				
LL=25 ENL=35	$CD3^+EM$	ENL	7.620	0.0409	354.5	3.095	0.0300 to 6.350
		LL	10.44				
	$CD4^+EM$	ENL	9.570	0.2184	414.5	2.200	-1.670 to 6.200
		LL	13.05				
	$CD8^+EM$	ENL	6.490	0.5037	456.5	1.100	-1.680 to 4.100
		LL	8.465				

P= probability (significant at $P \leq 0.05$); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 4. Median percentage of central memory T-cells expressed by $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the PBMCs of ENL cases and LL control patients before, during and after treatment

time	cell type	Patients	Median (%)	P	U	H-L	95 % Confidence interval
before LL=25 ENL=35	$CD3^+CM$	ENL	13.80	0.5354	453.5	1.100	-2.500 to 4.650
		LL	14.60				
	$CD4^+CM$	ENL	23.50	0.0015	268.5	-8.130	-12.20 to -3.560
		LL	14.40				
	$CD8^+EM$	ENL	1.220	< 0.0001	165.0	2.340	1.330 to 4.510
		LL	3.530				
during LL=25 ENL=35	$CD3^+CM$	ENL	9.770	0.0955	270.0	2.400	-0.6000 to 5.190
		LL	12.70				
	$CD4^+CM$	ENL	13.65	0.3444	312.0	3.370	-2.930 to 9.200
		LL	18.10				
	$CD8^+CM$	ENL	1.850	0.0696	261.5	0.9250	-0.090 to 1.940
		LL	2.450				
LL=25 ENL=35	$CD3^+CM$	ENL	13.50	0.6535	473.0	-1.000	-6.310 to 2.810
		LL	13.80				
	$CD4^+CM$	ENL	22.00	0.4868	454.5	-3.550	-12.90 to 5.000
		LL	19.35				
	CD8+CM	ENL	2.660	0.1829	407.0	-0.5600	-1.540 to 0.2100
		LL	2.015				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 5. Median percentage of terminally differentiated T-cells expressed by $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the PBMCs of ENL cases and LL control patients before, during and after treatment

time	cell type	Patients	Median (%)	P	U	H-L	95 % Confidence interval
before LL=25 ENL=35	$CD3^+$ EC	ENL	29.25	0.0062	299.0	-9.000	-16.30 to -2.700
		LL	20.00				
	$CD4^+$ EC	ENL	14.00	0.6417	465.0	-1.000	-5.400 to 3.310
		LL	11.66				
	$CD8^+$ EC	ENL	62.65	< 0.0001	147.5	-25.80	-33.20 to -17.30
		LL	39.50				
during LL=25 ENL=35	$CD3^+$ EC	ENL	43.35	0.8160	354.0	2.300	-8.600 to 10.80
		LL	37.10				
	$CD4^+$ EC	ENL	22.90	0.4953	327.5	-3.400	-13.40 to 4.500
		LL	17.90				
	$CD8^+$ EC	ENL	53.35	0.5225	330.0	-3.600	-14.10 to 6.500
		LL	50.20				
LL=25 ENL=35	$CD3^+$ EC	ENL	24.60	0.0489	360.0	8.650	0.0 to 17.10
		LL	35.30				
	$CD4^+$ EC	ENL	13.10	0.1425	397.0	3.900	-1.800 to 9.720
		LL	15.25				
	CD8+ EC	ENL	38.90	0.0006	304.0	14.40	3.900 to 23.80
		LL	55.20				

P= probability (significant at $P \leq 0.05$); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 6. Median percentage of naïve T-cells among $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the PBMCs of ENL cases and LL control patients before, during and after treatment

time	cell type	Patients	Median (%)	P	U	LH	95 % Confidence interval
before LL=25 ENL=35	$CD3^+$ NC	ENL	27.70	< 0.0001	111.0	26.50	19.60 to 31.73
		LL	59.48				
	$CD4^+$ NC	ENL	34.10	< 0.0001	116.0	25.60	17.16 to 33.50
		LL	61.54				
	$CD8^+$ NC	ENL	15.35	< 0.0001	105.5	31.06	23.20 to 37.70
		LL	50.50				
during LL=25 ENL=35	$CD3^+$ NC	ENL	31.80	0.7002	345.0	-2.100	-10.60 to 7.900
		LL	32.90				
	$CD4^+$ NC	ENL	45.15	0.2947	306.0	-4.150	-14.40 to 5.800
		LL	40.00				
	$CD8^+$ NC	ENL	35.45	0.8690	358.0	1.550	-9.400 to 13.50
		LL	38.10				
LL=25 ENL=35	$CD3^+$ NC	ENL	42.90	0.0409	354.5	-8.950	-16.60 to -0.200
		LL	33.00				
	$CD4^+$ NC	ENL	42.90	0.5786	465.0	-1.750	-10.80 to 6.400
		LL	41.75				
	CD8+ NC	ENL	51.50	0.0110	318.5	-14.40	-24.60 to -2.800
		LL	32.80				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 7. Median percentage of Memory T-cells expressed by $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the PBMCs of ENL cases before, during and after treatment

Cell type	Patients	Median (%)	P	U	HL	95% Confidence interval for HL
CD3+CD45RO+	1st	41.05	0.0010	354.0	-11.20	-17.60 to -4.300
	2nd	29.20				
CD3+CD45RO+	1st	41.05	0.0014	471.5	-9.800	-16.00 to -3.700
	3rd	31.15				
CD3+CD45RO+	2nd	29.20	0.7718	614.0	0.9000	-5.100 to 7.300
	3rd	31.15				
CD4+CD45RO+	1st	52.30	< 0.0001	164.0	-22.10	-28.60 to -14.60
	2nd	29.20				
CD4+CD45RO+	1st	52.30	0.0495	596.0	-8.750	-17.60 to 0.0
	3rd	45.00				
CD4+CD45RO+	2nd	29.20	0.0013	360.5	14.30	5.690 to 21.80
	3rd	45.00				
CD8+CD45RO+	1st	17.50	0.1259	504.5	-3.600	-8.300 to 0.9000
	2nd	12.55				
CD8+CD45RO+	1st	17.50	0.0516	562.0	-4.960	-9.660 to 0.7000
	3rd	12.85				
CD8+CD45RO+	2nd	12.55	0.4517	573.0	-1.335	-4.820 to 2.100
	3rd	12.85				

P= probability (significant at $P \leq 0.05$); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 8. Median percentage of activated $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the PBMCs of ENL cases before, during and after treatment

Cell type	Patients	Median (%)	P	U	H-L	95% Confidence interval for HL
CD3+CD62L-	1st	59.15	0.0176	431.5	-13.50	-24.60 to -2.200
	2nd	46.95				
CD3+CD62L-	1st	59.15	< 0.0001	233.0	-25.50	-31.60 to -17.80
	3rd	33.90				
CD3+CD62L-	2nd	29.65	0.1231	503.5	-10.70	-22.20 to 2.700
	3rd	33.90				
CD4+CD62L-	1st	50.65	< 0.0001	299.5	--17.75	-28.40 to -9.200
	2nd	29.65				
CD4+CD62L-	1st	50.65	< 0.0001	330.5	-20.00	-28.30 to -12.60
	3rd	29.35				
CD4+CD62L-	2nd	29.65	0.6141	595.0	-2.000	--9.800 to 6.500
	3rd	29.35				
CD8+CD62L-	1st	71.15	0.0378	457.0	-11.70	-24.00 to -0.9000
	2nd	59.50				
CD8+CD62L-	1st	71.15	< 0.0001	62.50	-34.20	-40.20 to -27.70
	3rd	34.45				
CD8+CD62L-	2nd	59.50	0.0007	347.0	-22.00	-33.30 to -10.20
	3rd	34.45				

P= probability (significant at $P \leq 0.05$); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 9. Median percentage of effector memory T cells expressed by $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the PBMCs of ENL cases before, during and after treatment

Cell type	Patients	Median (%)	P	U	HL	95% Confidence interval for HL
CD3+EM	1st	26.55	< 0.0001	256.0	-11.88	-16.74 to -6.110
	2nd	16.80				
CD3+EM	1st	26.55	< 0.0001	85.00	-20.00	-23.68 to -16.16
	3rd	7.620				
CD3+EM	2nd	16.80	< 0.0001	269.0	-8.200	-12.29 to -4.590
	3rd	7.620				
CD4+EM	1st	24.55	< 0.0001	223.0	-15.54	-20.30 to -9.660
	2nd	11.40				
CD4+EM	1st	24.55	< 0.0001	190.0	-16.23	-20.50 to -11.80
	3rd	9.570				
CD4+EM	2nd	11.40	0.5106	566.5	-1.080	-4.840 to 2.400
	3rd	9.570				
CD8+EM	1st	16.50	0.0024	375.5	-6.610	-10.53 to -2.390
	2nd	7.530				
CD8+EM	1st	16.50	0.0003	418.0	-7.505	-11.30 to -3.530
	3rd	6.490				
CD8+EM	2nd	7.530	0.4497	558.0	-0.8900	-3.400 to 1.840
	3rd	6.490				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 10. Median percentage of central memory T cells expressed by $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the PBMCs of ENL cases before, during and after treatment

Cell type	Patients	Median (%)	P	U	H-L	95% Confidence interval for HL
CD3+CM	1st	13.80	0.0014	362.5	-4.100	-6.610 to -1.400
	2nd	9.770				
CD3+CM	1st	13.80	0.7347	745.0	0.5400	-3.430 to 4.600
	3rd	13.50				
CD3+CM	2nd	9.770	0.0129	410.0	4.630	0.9000 to 9.490
	3rd	13.50				
CD4+CM	1st	23.50	0.0007	347.0	-9.035	-13.30 to -4.130
	2nd	13.65				
CD4+CM	1st	23.50	0.9281	770.5	-0.3500	-7.900 to 7.900
	3rd	22.00				
CD4+CM	2nd	13.65	0.0234	428.5	8.600	0.9000 to 17.60
	3rd	22.00				
CD8+CM	1st	1.220	0.0173	431.0	0.6500	0.1100 to 1.230
	2nd	1.850				
CD8+CM	1st	1.220	< 0.0001	374.0	1.320	0.6300 to 2.080
	3rd	2.660				
CD8+CM	2nd	1.850	0.0980	480.5	0.6500	-0.1100 to 1.580
	3rd	2.660				

P= probability (significant at $P \leq 0.05$); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 11. Median percentage of effector T- cells expressed by $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the PBMCs of ENL cases before, during and after treatment

Cell type	Patients	Median (%)	P	U	H-L	95% Confidence interval for HL
CD3+EC	1st	29.25	0.0037	387.0	11.10	3.000 to 19.10
	2nd	43.35				
CD3+EC	1st	29.25	0.3862	691.0	-3.165	-9.500 to 4.900
	3rd	24.60				
CD3+EC	2nd	43.35	0.0024	364.5	-12.80	-22.00 to -4.900
	3rd	24.60				
CD4+EC	1st	14.00	0.0389	458.0	7.400	0.4800 to 15.80
	2nd	22.90				
CD4+EC	1st	14.00	0.4716	706.0	-1.625	-6.020 to 2.890
	3rd	12.1				
CD4+EC	2nd	22.90	0.0200	423.5	-8.185	--16.40 to -0.9800
	3rd	12.1				
CD8+EC	1st	62.65	0.0267	445.0	445.0	-21.40 to -1.300
	2nd	53.35				
CD8+EC	1st	62.65	< 0.0001	310.0	-24.20	-33.10 to -15.50
	3rd	38.90				
CD8+EC	2nd	53.35	0.0223	427.0	-12.40	-22.40 to -1.700
	3rd	38.90				

P= probability (significant at $P \leq 0.05$); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 12. Median percentage of naïve $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the PBMCs of ENL cases before, during and after treatment

Cell type	Patients	Median (%)	P	U	H-L	95% Confidence interval for HL
CD3+NC	1st	27.70	0.1776	520.5	5.800	-2.470 to 13.30
	2nd	31.80				
CD3+NC	1st	27.70	< 0.0001	293.5	19.60	11.30 to 26.19
	3rd	42.90				
CD3+NC	2nd	31.80	0.0030	370.0	13.60	4.700 to 22.10
	3rd	42.90				
CD4+NC	1st	34.10	0.0011	357.0	12.10	5.300 to 20.20
	2nd	45.15				
CD4+NC	1st	34.10	0.0022	472.0	13.80	5.700 to 20.80
	3rd	40.4				
CD4+NC	2nd	45.15	0.7068	591.0	1.450	-8.700 to 10.50
	3rd	40.40				
CD8+NC	1st	15.35	0.0003	324.0	17.10	7.900 to 26.30
	2nd	35.45				
CD8+NC	1st	15.35	< 0.0001	237.0	30.90	21.20 to 38.40
	3rd	51.50				
CD8+NC	2nd	35.45	0.0176	419.5	11.10	1.800 to 21.80
	3rd	51.50				

P= probability (significant at $P \leq 0.05$); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 13. IL-17 Producing cells in the PBMCs of patients with ENL cases before, during and after treatment

Cell type	Patients	Median (%)	P	U	HL	95% Confidence interval for HL
IL-17 producing T cells	1st	23.10	0.0006	512.5	-6.800	-10.50 to -2.700
	2nd	16.40				
	1st	23.10	< 0.0001	440.5	-9.325	-13.30 to -5.700
	3rd	13.25				
	2nd	16.40	0.0859	669.5	-2.700	-5.900 to 0.4000
	3rd	13.25				
IL-17 producing T lymphocytes	1st	26.45	< 0.0001	361.0	-9.500	-12.90 to -5.800
	2nd	15.40				
	1st	26.45	< 0.0001	346.5	-11.85	-15.40 to -8.300
	3rd	12.70				
	2nd	15.40	0.1252	689.5	-2.800	-6.100 to 0.8300
	3rd	12.70				
CD4+CD161+	1st	20.35	< 0.0001	395.0	-6.870	-9.730 to -3.640
	2nd	14.40				
	1st	20.35	0.2265	902.5	-1.600	-4.800 to 1.100
	3rd	18.60				
	2nd	14.40	< 0.0001	425.5	5.100	2.640 to 7.540
	3rd	18.60				
CD8+CD161+	1st	7.120	0.1967	750.0	-1.170	-2.960 to 0.5700
	2nd	6.140				
	1st	7.120	0.4511	918.0	0.6250	-1.050 to 2.500
	3rd	7.380				
	2nd	6.140	0.0445	638.0	1.860	0.01000 to 3.680
	3rd	7.380				

Appendix 7: Tables for B-cell results

Total 1. B Lymphocytes ($CD19^+$) and matured B –cells ($CD19^+CD10^-$) (ENL vs LL)

Table 2. Total nave B Lymphocytes (CD19+ $CD10^-CD27^-CD21^+$) expression (ENL vs LL)

Table 3. Expression of activated memory (AM) B-cells ($CD19^+CD10^-CD27^+CD21^-$) and tissue like memory (TLM) B –cells ($CD19^+CD10^-CD27^-CD21^-$) (ENL vs LL)

Table 4. Total B Lymphocytes ($CD19^+$) and matured B-cells ($CD19+CD10^-$) expression (ENL before and after treatment)

Table 5. Total nave (NB) B Lymphocytes ($CD19^+CD10^-CD27^-CD21^+$) and resting memory (RM) B -cells ($CD19^+CD10^-CD27^+CD21^+$) expression (ENL before and after treatment)

Table 6. Expression of activated memory (AM) B-cells ($CD19^+CD10^-CD27^+CD21^-$) and tissue like memory (TLM) B -cells ($CD19^+CD10^-CD27^-CD21^-$) (ENL before and after treatment)

Table 1. Total B Lymphocytes ($CD19^+$) and matured B –cells ($CD19^+CD10^-$) production in the PBMCs of ENL cases and LL patient controls before, during and after treatment of ENL cases with prednisolone.

time	Cell type	patients	Median (%)	P	U	LH	95% confidence interval
Before Treatment	CD19+B cells	ENL	9.490	0.3032	198.0	1.845	-2.100 to 6.960
		LL	11.55				
	CD19+CD10- B cells	ENL	98.00	0.9132	238.0	0.0	-1.300 to 0.6000
		LL	97.25				
During Treatment	CD19+B cells	ENL	9.890	0.4396	53.00	2.820	-3.430 to 6.520
		LL	13.90				
	CD19+CD10- B cells	ENL	98.95	0.1366		-0.50	-1.700 to 0.2000
		LL	98.00				
After Treatment	CD19+B cells	ENL	5.700	0.0004	96.00	6.020	2.800 to 9.000
		LL	11.95				
	CD19+CD10- B cells	ENL	97.35	0.5333	215.0	0.200	-0.6000 to 1.200
		LL	98.10				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 2. Total nave B Lymphocytes (CD19+ $CD10^-CD27^-CD21^+$) and resting memory B –cells ($CD19^+CD10^-CD27^+CD21^+$) expression in the PBMCs of ENL cases and LL patient controls before, during and after treatment of ENL cases with prednisolone

time	Cell type	patients	Median (%)	P	U	LH	confidence interval
Before Treatment	NB	ENL	76.00	0.0377	153.5	6.750	0.6000 to 15.30
		LL	84.60				
	RM	ENL	5.840	0.3731	204.0	-0.7300	-3.040 to 0.8600
		LL	4.760				
During Treatment	NB	ENL	64.65	0.8664	63.00	1.150	-14.80 to 15.60
		LL	64.00				
	RM	ENL	15.25	0.0316	31.00	-6.950	-16.04 to -0.5000
		LL	8.630				
After Treatment	NB	ENL	74.30	0.7759	229.5	-1.150	-10.90 to 8.700
		LL	71.55				
	RM	ENL	3.720	0.1301	177.0	-1.155	-3.190 to 0.2900
		LL	1.795				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 3. Expression of activated memory (AM) B-cells ($CD19^{+}CD10^{-}CD27^{+}CD21^{-}$) and tissue like memory (TLM) B –cells ($CD19^{+}CD10^{-}CD27^{-}CD21^{-}$) in the PBMCs of ENL cases and LL patient controls before, during and after treatment of ENL cases with prednisolone.

time	Cell type	patients	Median (%)	P	U	LH	confidence interval
Before Treatment	AM	ENL	2.120	0.3254	200.0	-0.4950	-1.990 to 0.5200
		LL	1.425				
	TLM	ENL	9.280	0.0246	146.5	-3.725	-8.030 to -0.3100
		LL	4.815				
During Treatment	AM	ENL	3.825	0.3429	50.00	0.7900	-1.710 to 3.350
		LL	4.410				
	TLM	ENL	13.20	0.1326	41.00	6.390	-1.580 to 17.50
		LL	20.00				
After Treatment	AM	ENL	2.960	0.9487	239.0	-0.0750	-1.470 to 2.020
		LL	3.005				
	TLM	ENL	10.00	0.0792	167.0	-2.200	-4.740 to 0.2000
		LL	7.295				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 4. Total B Lymphocytes ($CD19^{+}$) and matured B-cells ($CD19+CD10^{-}$) production in the PBMCs of ENL cases before, during and after treatment with prednisolone.

Cell type	Patients	Median (%)	P	U	H-L	95% Confidence interval for HL
CD19+ B -cells	1^{st}	9.490	0.7019	149.0	0.4750	-5.440 to 4.800
	2^{nd}	9.890				
CD19+ B- cells	1^{st}	9.490	0.0231	184.5	-3.925	-7.640 to -0.9300
	3^{rd}	5.700				
CD19+ B-cells	2^{nd}	9.890	0.0205	68.00	-4.585	-7.820 to -0.5600
	3^{rd}	5.700				
CD19+CD10-	1st	98.00	0.0066	74.50	1.000	0.4000 to 2.400
	2nd	98.95				
CD19+CD10-	1st	98.00	0.6791	276.0	0.2000	-1.000 to 1.100
	3rd	97.35				
CD19+CD10-	2nd	98.95	0.0377	74.50	-0.9500	-2.200 to -0.1000
	3rd	97.35				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 5. Total nave (NB) B Lymphocytes ($CD19^{+}CD10^{-}CD27^{-}CD21^{+}$) and resting memory (RM) B -cells ($CD19^{+}CD10^{-}CD27^{+}CD21^{+}$) expression in the PBMCs of ENL cases before, during and after treatment with prednisolone.

Cell type	Patients	Median (%)	P	U	HL	95% Confidence interval for HL
NB	Before	76.00	0.0164	84.00	-14.05	-25.10 to -2.500
	During	64.65				
NB	Before	76.00	0.2482	239.0	-5.100	-13.10 to 3.500
	After	74.30				
NB	During	64.65	0.2569	100.0	7.400	-4.700 to 21.40
	After	74.30				
RM	Before	5.840	0.0008	56.50	8.405	3.700 to 15.87
	During	15.25				
RM	Before	5.840	0.0250	186.0	-2.185	-4.140 to -0.3600
	After	3.720				
RM	During	15.25	< 0.0001	56.50	8.405	3.700 to 15.87
	After	3.720				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Table 6. Expression of activated memory (AM) B-cells ($CD19^{+}CD10^{-}CD27^{+}CD21^{-}$) and tissue like memory (TLM) B -cells ($CD19^{+}CD10^{-}CD27^{-}CD21^{-}$) in the PBMCs of ENL cases before, during and after treatment with prednisolone.

Cell type	Patients	Median (%)	P	U	HL	95% Confidence interval for HL
AM	1^{st}	2.120	0.4386	136.0	0.7050	-1.190 to 2.630
	2^{nd}	3.825				
AM	1^{st}	2.120	0.8538	287.5	0.1150	-1.360 to 1.420
	3^{rd}	2.960				
AM	2^{nd}	3.825	0.3309	104.5	-0.6950	-2.620 to 1.000
	3^{rd}	2.960				
TLM	1st	9.280	0.1662	116.0	3.900	-2.000 to 7.820
	2nd	13.20				
TLM	1st	9.280	0.8695	288.5	-0.3000	-4.900 to 2.840
	3rd	10.00				
TLM	2nd	13.20	0.0283	71.50	-3.840	-8.280 to -0.4000
	3rd	10.00				

P= probability (significant at P≤0.05); U= Mann-Whitney U; H L= Hodges-Lehmann

Appendix 8: Cytokine ELISA Standard Curves and Tables

Table 1. Comparison of the levels of *in vitro* cytokine production in culture supernatants of PBMCs from patients with ENL and LL controls before and after treatment

Time point	Cytokine	group	*mean ± SE	t, df	95% CI	P-value
Before treatment	TNF-α	ENL	83.56 ± 18.82	t=2.629 df=67	-112.8 to -15.44	0.0106
		LL	19.42 ± 10.44			
	IFN-γ	ENL	1361 ± 309.60	t=2.456 df=66	-1960 to -202.2	0.0167
		LL	280.1 ± 269.80			
	IL-1β	ENL	29.07 ± 7.03	t=0.3989 df=75	-29.71 to 19.80	0.6911
		LL	24.12 ± 10.99			
	IL-17A	ENL	51.38 ± 14.26	t=3.474 df=48	-78.44 to -20.93	0.0011
		LL	1.698 ± 1.07			
	IL-6	ENL	2194 ± 535.9	t=3.8531 df=73	-778.6 to -23.60	0.0014
		LL	1454 ± 686.7			
	IL-8	ENL	30775 ± 3402	t=2.968 df=70	-27713 to -408.7	0.0258
		LL	19463 ± 4714			
	IL-10	ENL	18.59 ± 4.054	t=5.277 df=67	64.67 to 143.4	< 0.0001
		LL	122.6 ± 23.18			
During treatment	TNF-α	ENL	10.74 ± 2.786	t=1.621 df=62	-6.100 to 58.43	0.1101
		LL	36.91 ± 17.44			
	IFN-γ	ENL	340.4 ± 119.6	t=2.720 df=62	132.6 to 1767	0.00904
		LL	1158 ± 549.2			
	IL-1β	ENL	11.30 ± 3.736	t=0.6762 df=62	-15.88 to 7.850	0.5014
		LL	7.287 ± 4.714			
	IL-17A	ENL	7.119 ± 3.206	t=0.4886 df=47	-15.18 to 24.91	0.6274
		LL	11.99 ± 9.609			
	IL-6	ENL	1219 ± 376.2	t=0.06602 df=63	-1234 to 1155	0.9476
		LL	1179 ± 474.7			
	IL-8	ENL	31271 ± 3315.0	t=2.373 df=63	-22481 to -1929	0.0207
		LL	19066 ± 3969.0			
	IL-10	ENL	13.03 ± 2.55	t=0.3470 df=62	-7.199 to 10.22	0.7298
		LL	14.55 ± 3.737			

* pg/mlL

Time point	Cytokine	group	*mean ± SE		95% CI	P-value
After treatment	TNF-α	ENL	25.37 ± 8.88	t=0.4275 df=48	-38.72 to 25.15	0.6710
		LL	18.58 ± 13.17			
	IFN-γ	ENL	328.2 ± 190.30	t=0.8521 df=55	-441.3 to 1094	0.3979
		LL	654.6 ± 336.40			
	IL-1β	ENL	9.598 ± 2.30	t=2.112 df=54	3.140 to 120.5	0.0393
		LL	71.42 ± 30.25			
	IL-17A	ENL	17.64 ± 7.96	t=2.098 df=48	-31.09 to -1.568	0.0377
		LL	2.876 ± 1.63			
	IL-6	ENL	1567 ± 524.00	t=0.1636 df=54	-1568 to 1846	0.8707
		LL	1706 ± 679.80			
	IL-8	ENL	28864 ± 3987.00	t=2.933 df=53	-21490 to -395.7	0.0362
		LL	18317 ± 3711.00			
	IL-10	ENL	87.78 ± 22.49	t=3.539 df=54	-129.8 to -35.93	0.0008
			4.908 ± 2.160			

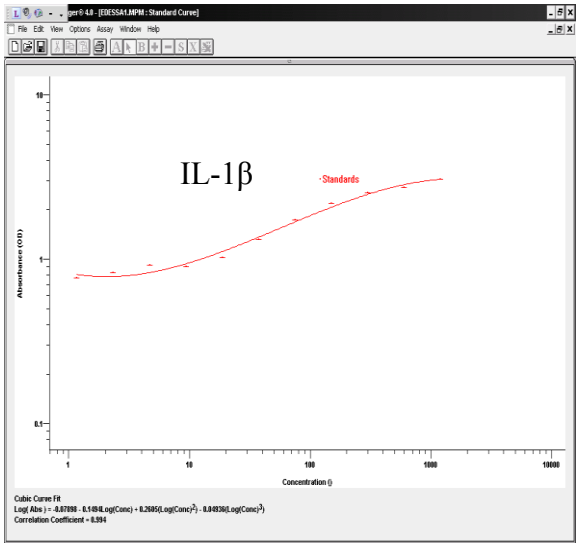
* pg/mlL

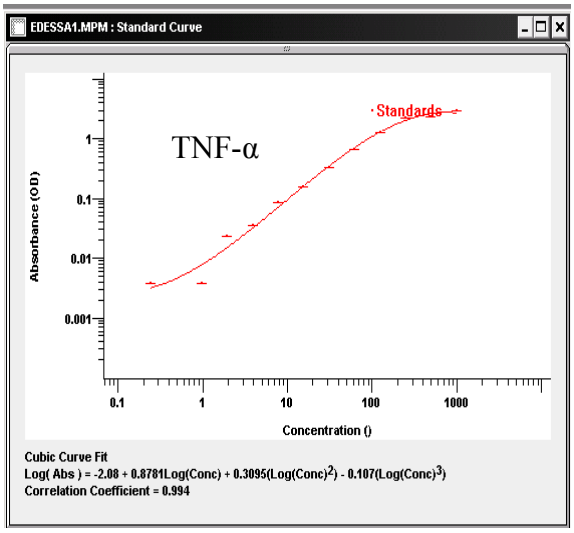
Table 2. Comparison of the levels of *in vitro* cytokine production in culture supernatants of PBMCs from patients with ENL before and after treatment

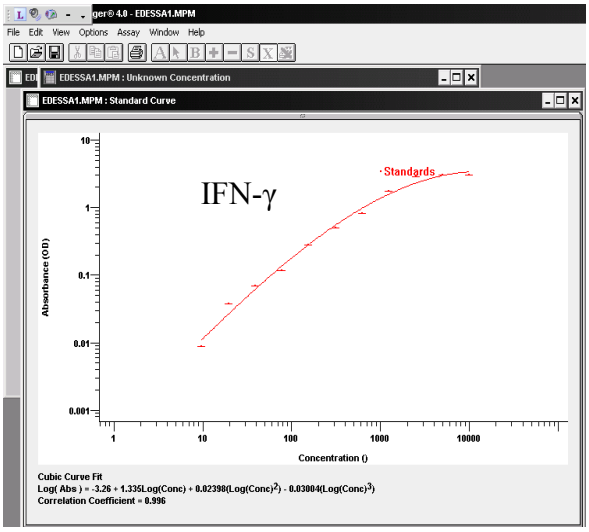
Cytokine	Time point	*mean ± SE	t, df	95% CI	P-value
TNF-α	Before treatment	83.56 ± 18.82	t=3.924 df=34	-130.4 to -41.42	0.0004
	During Treatment	10.74 ± 2.79			
	Before treatment	83.56 ± 18.82	t=3.397 df=24	-155.4 to -37.93	0.0024
	After treatment	25.37 ± 8.88			
	During Treatment	10.74 ± 2.79	t=1.805 df=24	-1.933 to 28.94	0.0836
	After treatment	25.37 ± 8.88			
IFN-γ	Before treatment	1361 ± 309.6	t=2.957 df=37	-1846 to -344.8	0.0054
	During Treatment	340.4 ± 119.6			
	Before treatment	1361 ± 309.6	t=3.027 df=28	-2424 to -467.5	0.0053
	After treatment	328.2 ± 190.3			
	During Treatment	340.4 ± 119.6	t=0.3971 df=28	-638.2 to 430.9	0.6943
	After treatment	328.2 ± 190.3			
IL-1β	Before treatment	29.07 ± 7.026	t=2.164 df=35	-40.90 to -1.304	0.0374
	During Treatment	11.30 ± 3.736			
	Before treatment	29.07 ± 7.026	t=2.406 df=28	-42.92 to -3.450	0.0230
	After treatment	9.598 ± 2.301			
	During Treatment	11.30 ± 3.736	t=0.5001 df=27	-12.60 to 7.660	0.6211
	After treatment	9.598 ± 2.301			
IL-17A	Before treatment	51.38 ± 14.26	t=3.290 df=24	-72.04 to -16.49	0.0031
	During Treatment	7.119 ± 3.206			
	Before treatment	51.38 ± 14.26	t=2.400 df=24	-62.76 to -4.732	0.0245
	After treatment	17.64 ± 7.956			
	During Treatment	7.119 ± 3.206	t=1.226 df=24	-7.193 to 28.23	0.2322
	After treatment	17.64 ± 7.956			

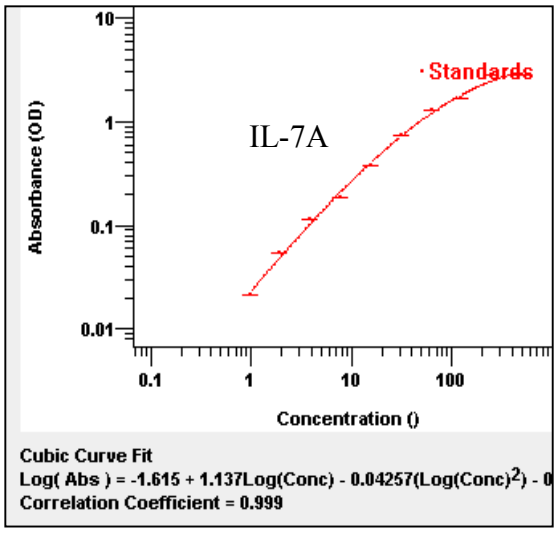
Cytokine	Time point	*mean ± SE	t, df	95% CI	P-value
IL-6	Before treatment	2194 ± 535.9	t=1.637 df=36	-2733 to 291.3	0.1103
	During Treatment	1219 ± 376.2			
	Before treatment	2194 ± 535.9	t=1.115 df=28	-3008 to 887.6	0.2743
	After treatment	1567 ± 524.0			
	During Treatment	1219 ± 376.2	t=0.2519 df=28		0.8029
	After treatment	1567 ± 524.0		-1399 to 1791	
IL-8	Before treatment	30775 ± 3402	t=0.2348 df=36	-8916 to 11251	0.8157
	During Treatment	31271 ± 3315			
	Before treatment	30775 ± 3402	t=0.7162 df=27	-19591 to 9454	0.4800
	After treatment	28864 ± 3987			
	During Treatment	31271 ± 3315	t=0.8823 df=27	-14416 to 5746	0.3854
	After treatment	28864 ± 3987			
IL-10	Before treatment	18.59 ± 4.054	t=1.591 df=36	-16.67 to 2.013	0.1204
	During Treatment	13.03 ± 2.545			
	Before treatment	18.59 ± 4.054	t=2.528 df=28	11.79 to 112.6	0.0174
	After treatment	87.78 ± 22.49			
	During Treatment	13.03 ± 2.545	t=3.244 df=28	27.54 to 121.9	0.0030
	After treatment	87.78 ± 22.49			

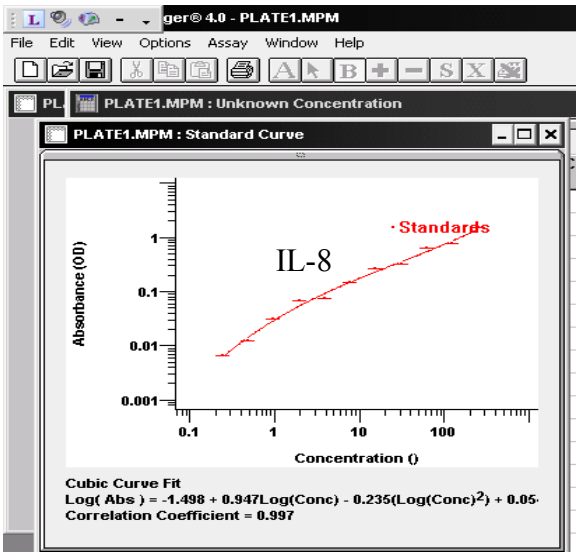
ELISA standard curves

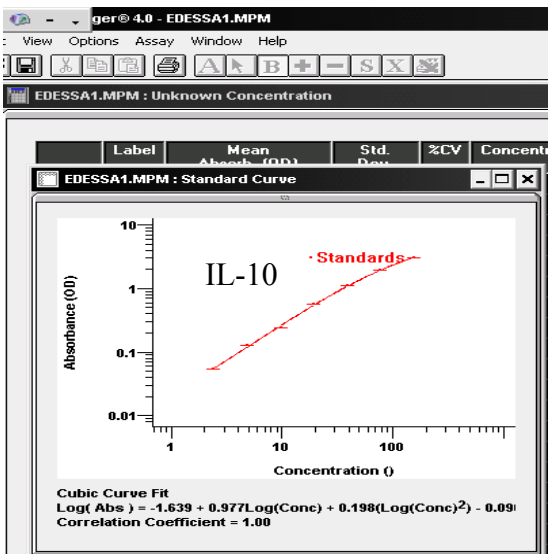




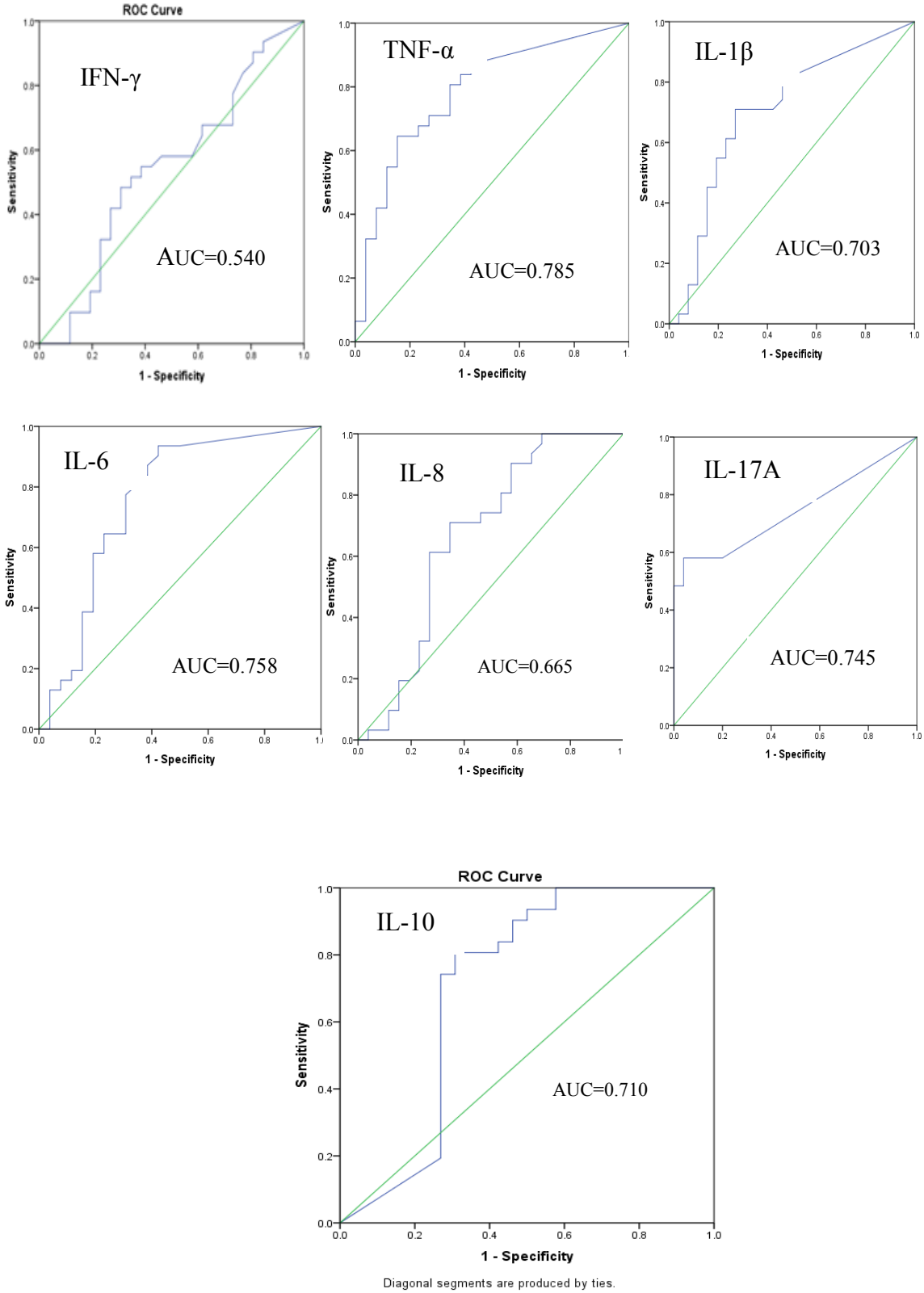








ROC for individual cytokines



Appendix 9: Circulating C1q ELISA Tables

Table 1. Comparison of circulating plasma C1q in patients with ENL and LL controls

Time point	Group	Mean ± SE (pg/mL)	t, df	95% CI	P
Before treatment	ENL	11698 ± 618.3	t=4.201 df=59	4902 to 13822	< 0.0001
	LL	21059 ± 2382			
After treatment	ENL	22287 ± 2154	t=0.4900 df=60	-4421 to 7290	0.6259
	LL	23721 ± 1886			
Before treatment	ENL	11698 ± 618.3	t=2.255 df=39	439.5 to 8090	0.0298
	HC	15962 ± 3011			
	LL	21059 ± 2382	t=1.029 df=32	-15184 to 4990	0.3110
	HC	15962 ± 3011			

Table 2. Comparison of circulating plasma C1q in patients with ENL before and after treatment

Time point	Mean ± SE (pg/mL)	t, df	95% CI	P
ENL 1st	11698 ± 618.3	t=5.071 df=33	6341 to 14838	< 0.0001
ENL 3nd	22287 ± 2154			
LL 1st	21059 ± 2382 N=27	t=0.6881 df=26		0.4975
LL 3rd	23721 ± 1886			

Table 8.1. Plasma anti PGL-1, LAM and Ag85 titres of patients with ENL and LL controls before and after treatment

ENL versus LL						
Antibody	time point	Pt	Mean ± SE	t, df	95% CI	P-value
PGL-1	Before	ENL	1.430 ± 0.1281	t=0.4653 df=50	-0.4711 to 0.2939	0.6437
		LL	1.341 ± 0.1415			
	After	ENL	1.183 ± 0.1333	t=5.268 df=49	0.5617 to 1.255	< 0.0001
		LL	2.091 ± 0.1081			
Ag85	before	ENL	1.154 ± 0.1218	t=0.1136 df=50	-0.3864 to 0.3450	0.9100
		LL	1.134 ± 0.1360			
	after	ENL	0.9713 ± 0.1164	t=3.508 df=49	0.2411 to 0.8880	0.0010
		LL	1.536 ± 0.1108			
LAM	before	ENL	1.191 ± 0.09790	t=0.4876 df=50	-0.3811 to 0.2322	0.6280
		LL	1.116 ± 0.1183			
	after	ENL	1.095 ± 0.08101	t=4.258 df=49	0.2627 to 0.7323	< 0.0001
		LL	1.592 ± 0.08426			
ENL before and after treatment						

Appendix 10: Conferences and publication

Conferences

World Immune Regulation Meeting (WIRM)

March 16-19, 2016, Davos Switzerland

Titles of the abstracts

i. T-cell regulation in Erythema Nodosum Leprosy

Edessa Negera[1,2], Anastasia Polycarpou[1], Kidist Bobosha[2], Steven Walker[1], Yonas Bekele[2], Birtukam Endale[2], Munir Hussien[2], Rawleigh Howe[2], Abraham Aseffa[2], Hazel M. Dockrell[1], Diana Lockwood[1]

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ii. Memory T-cells and Lipid profiles of leprosy Spectrum

Edessa Negera[1,2], Anastasia Polycarpou[1], Kidist Bobosha[2], Steven Walker[1], Yonas Bekele[2], Birtukam Endale[2], Munir Hussien[2], Rawleigh Howe[2], Abraham Aseffa[2], Hazell M. Docrell[1], Diana Lockwood[1]

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Accepted abstracts: International Leprosy congress, Beijing China

September 16-21, 2016

i. A Case-control Study Investigating T-cell Regulation in Erythema Nodosum Leprosum

Edessa Negera[1,2], Anastasia Polycarpou[1], Kidist Bobosha[2], Steven Walker[1], Yonas Bekele[2], Birtukam Endale[2], Munir Hussien[2], Rawleigh Howe[2], Abraham Aseffa[2], Hazell M. Docrell[1], Diana Lockwood[1]

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Abstract

Background: Erythema Nodosum Leprosum (ENL) is an immune-mediated inflammatory complication which causes high morbidity in affected leprosy patients.

Objective: This study investigated the immune regulation in patients with Erythema Nodosum Leprosum

Methods: A follow-up nested case-control study design was used to recruit a 46 untreated patients with ENL reactions and 31 non-reactional lepromatous leprosy (LL) patient controls at ALERT hospital, Ethiopia. Blood samples were obtained before, during and after treatment. Peripheral blood mononuclear cells (PBMCs) were isolated and used for immunophenotyping of regulatory T-cells by flow cytometry. Five markers were used to define $CD4^+$ and $CD8^+$ regulatory T-cells. The gene expression for FoxP3 in blood and tissue samples was used to supplement the flow cytometry data. Clinical and histopathological data were also obtained for each patient. All patients were followed for 28 weeks.

Results: Patients with ENL reactions had significantly lower (1.67%) $CD4^+$ regulatory T-cells than LL patient controls (3.79%) before treatment. After treatment, $CD4^+$Tregulatory T-cells in the two groups were not significantly different. $CD8^+$ regulatory T-cells in both groups before and after treatment were not significantly deferent. Patients with ENL reactions had higher $CD4^+$ Tells and $CD4^+/CD8^+$ T-cells ratio than lepromatous leprosy patient controls before treatment. The CD25 expression on $CD4^+$ and $CD8^+$ T-cells were not significantly different between the two groups suggesting that CD25 expression is not associated with ENL reactions while FoxP3 expression on $CD4^+$ T-cells was found to be associated with the reaction. The mRNA expression in blood and tissue samples for FOXP3 was significantly lower in patients with ENL reaction than in LL patient controls before treatment. After prednisolone treatment, the mRNA expression for *FOXP3* in the blood samples from patients with ENL reaction did not show significant change while it is significantly increased in skin biopsy samples. It is possible that there is local immune regulation at the site of reactional lesions in these patients. We found that corticosteroid treatment of patients with ENL reactions is associated with suppression of $CD4^+$ T-cells but not $CD8^+$ T-cells.

Conclusions: Our findings suggest that Erythema Nodosum Leprosum reaction is associated with reduced regulatory T-cells and increased $CD4^+/CD8^+$ T-cells ratio and this immune imbalance could lead to the initiation of ENL reaction in patients with leprosy.

ii. New Insight into the Pathogenesis of Erythema Nodosum Leprosum: Is there a Role of Activated Memory T-cells?

Edessa Negera[1,2], Anastasia Polycarpou[1], Kidist Bobosha[2], Steven Walker[1], Yonas Bekele[2], Birtukam Endale[2], Munir Hussien[2], Rawleigh Howe[2], Abraham Aseffa[2], Hazel M. Dockrell[1], Diana Lockwood[1]

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Background: Memory T-cells, particularly, effector memory T-cells are implicated in the pathogenesis of inflammatory diseases and may contribute to tissue injury and disease progression. Although Erythema Nodosum Leprosum (ENL) is an inflammatory complication, of leprosy, the role of memory T-cell subsets has never been studied in this patient group.

Objective: This study investigated the kinetics of memory T-cell subsets in patients with ENL before and after corticosteroid treatment.

Methods: A case -control study design was used and a total of 35 untreated patients with ENL reactions and 25 non-reactional LL patient controls were recruited at ALERT hospital, Ethiopia. Venous blood samples were obtained before, during and after treatment from each patient. Peripheral blood mononuclear cells (PBMCs) were isolated and used for immunophenotyping of T-cell activation and memory T-cell subsets by flow cytometry. The kinetics of these immune cells in patients with ENL reactions before and after treatment were compared with lepromatous leprosy control patients (LL) as well as within patients with ENL cases at different time points.

Results: The median percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells expressing activated T-cells were significantly higher in the PBMCs from patients with ENL than from LL controls before treatment. The median percentage of central and activated memory T-cells were significantly increased in patients with ENL reaction compared to LL patient controls before treatment. Interestingly, patients with ENL reactions had a lower percentage of naïve T-cells (28.0%) compared to LL patient controls (60.0%), however, after corticosteroid treatment, patients with ENL cases had a higher median percentage of naïve T-cells (43.0%) than LL patient controls (33.0%). The median

percentage of activated T-cells (effector memory T-cells and terminally differentiated T-cells) were significantly increased in patients with ENL reactions (59.2%) before treatment compared to after treatment (33.9%).

Conclusions: This is the first work which has shown T-cell activation and the different subsets of memory T-cells in untreated patients with ENL reactions. Consequently, this study delineates the role of T-cell activation in the pathogenesis of ENL reaction and challenges the long-standing dogma of immune-complex as a sole etiology of ENL reaction.

iii. Patterns of Cytokine Expression in the Blood and Skin Tissue samples from Patients with Erythema Nodosum Leprosum: The influence of Prednisolone Treatment

Edessa Negera[1,2], Anastasia Polycarpou[1], Kidist Bobosha[2], Steven Walker[1], Yonas Bekele[2], Birtukam Endale[2], Munir Hussien[2], Rawleigh Howe[2], Abraham Aseffa[2], Hazel M. Dockrell[1], Diana Lockwood[1]

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Background: Erythema nodosum leprosum (ENL) is a systemic inflammatory complication occurring mainly in patients with lepromatous leprosy (LL) and borderline lepromatous (BL) leprosy. Prednisolone is used for the treatment of patients with ENL, although it is not efficacious in preventing reoccurrences. This is the first study with good experimental design examining the effect of prednisolone on the kinetics of inflammatory cytokines in patients with ENL reactions.

Objective: This study described the kinetics of pro/inflammatory and regulatory cytokines production and gene expression in patients with ENL reactions before and after prednisolone treatment.

Methods: A follow-up nested case-control study design was used to recruit 46 untreated patients with ENL reactions and 31 non-reactional LL patient controls at ALERT hospital, Ethiopia. Blood and skin biopsy samples were obtained from each patient before and after treatment. To investigate the *in vitro* cytokine response to *M.leprae*, PBMCs from patients with ENL and LL controls were cultured with *M.leprae* whole-cell sonicates (MLWCS) in the presence of Phytohaemagglutinin (PHA) or left unstimulated for 6 days. The supernatants were collected and used for the Enzyme-linked immuno-absorbent assay (ELISA). To investigate the cytokine gene expression, mRNA was isolated from whole blood and skin biopsies and then reverse transcribed into cDNA. The mRNA copy numbers were quantified on the Light

Cycler using real-time PCR assays specific to TNF-α, IFN-γ, IL-β, TGF-β, IL-17A, IL-6, IL-8, IL-10 and HUPO.

Results: The *in vitro* production of the cytokines: TNF-α, IFN-γ, IL-1β and IL-17A were significantly increased in untreated patients with ENL reactions before treatment. However, IL-10 production was significantly lower in untreated patients and significantly increased after treatment. The *in vitro* production of IL-6 and IL-8 in patients with ENL reactions did not show statistically significant difference before and after prednisolone treatment. The mRNA expression in blood and skin biopsy samples for TNF-α, IFN-γ, IL-1β, IL-6 and IL-17A significantly decreased in patients with ENL reactions after treatment. IL-10 mRNA gene expression was significantly increased both in blood and tissue samples after treatment.

Conclusions: Our findings suggest that prednisolone modulates the pro/inflammatory cytokines studied here either directly or through suppressing the immune cells producing these inflammatory cytokines which need further confirmation.

iv. **Clinico-Pathological Features of Erythema Nodosum Leprosum: A case-control Study at ALERT Hospital, Ethiopia**.

Edessa Negera[1,2], Anastasia Polycarpou[1], Kidist Bobosha[2], Steven Walker[1], Yonas Bekele[2], Birtukam Endale[2], Munir Hussien[2], Rawleigh Howe[2], Abraham Aseffa[2], Hazel M. Dockrell[1], Diana Lockwood[1]

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Background: Leprosy reactions are a significant cause of morbidity. Erythema nodosum leprosum (ENL) is an immunological complication affecting approximately 50% of patients with lepromatous leprosy (LL) and 10% of borderline lepromatous (BL) leprosy. ENL is associated with skin lesions, neuritis, arthritis, dactylitis, eye inflammation, osteitis, orchitis, lymphadenitis and nephritis. The treatment of ENL requires immunosuppression, which is often required for prolonged periods of time and may lead to serious adverse effects.

Objective: We described the clinico-pathological features of patients with ENL before and after prednisolone treatment

Materials and methods: A case–controls study was performed at ALERT hospital, Ethiopia. Forty-six patients with ENL and 30 LL controls were enrolled to the study and followed for 28 weeks. Clinical futures were systematically documented at each time point on specifically prepared forms. Blood and skin biopsy samples were obtained from each patient. Laboratory and histopathological investigations were used to supplement the clinical data

Results: Pain was the commonest symptoms reported (98%) by patients with ENL. About 80% of them had reported skin pain and more than 70% had a nerve and joint pains during enrolment. About 40% of these patients developed chronic ENL. The morphology of cutaneous lesions showed that 95.7% individuals had nodular lesions. More than half (52.2%) of patients with ENL had old nerve function impairment (NFI) while 13% had new NFI at the time of enrolment. Facial and limb oedema were the most reported organs involved in ENL reactions. Before treatment dermal neutrophil infiltration was diagnosed in 58.8% of patients with ENL compared to 14.3% in LL controls. Only 14.7% of patients showed evidence of vasculitis.

Conclusion: The most frequent site of pain due to ENL in our study was the skin which is explained by the fact that 95% of patients with ENL cases had skin lesions. More diverse cutaneous manifestations of ENL are documented in this study than usually used in case definition. Whenever present, flat granular polymorphonuclear cells infiltration with perivascular lymphocyte infiltration were diagnosed as defining features of ENL lesions. The finding of chronic ENL in 40% of patients with ENL underline that these patients require corticosteroid treatment for extended periods. This implies that chronic ENL continue to be a burden for the health intuitions. Households affected by chronic ENL face significant economic burden and at risk of being pushed further into poverty.

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BMC Health Services Research

RESEARCH ARTICLE Open Acces

Performance of general health workers in leprosy control activities at public health facilities in Amhara and Oromia States, Ethiopia

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Abstract

Background: Leprosy is a chronic infectious disease of public health importance and one of the leading causes of permanent physical disability. Nevertheless, the drop in prevalence following multidrug therapy has resulted in the neglect of leprosy. The annual incidence of leprosy has remained the same in Ethiopia since decades with more than 76 % of the reported new cases coming from Oromia and Amhara Regional States. This study was aimed to assess the knowledge, attitude and skill of general health workers in leprosy control activities at public health facilities in Oromia and Amhara Regional States.

Methods: A cross-sectional study was conducted from September 2011 to February 2012 at different public health facilities in selected eight zones in Oromia and Amhara Regional States. A multistage sampling method was used to obtain representative samples. High and low endemic zones for leprosy were included in the study in both regional states. Data were collected from general health workers through a structured self-administered questionnaire and at on-site assessment of their performance. Baseline socio-demographic data, health workers' attitude towards leprosy and their knowledge and skill in the management of leprosy were assessed. Bloom's cut off point was used to describe the knowledge and practical skills of the respondents while Likert's scale was used for attitude assessment.

Result: A total of 601 general health workers responsible for leprosy control activities at public health facilities were included in knowledge and attitude assessment and 83 of them were subjected to practical evaluation, with on-site observation of how they handle leprosy patients. These included medical doctors (4 %), health officers and nurses with Bachelor degree in Science (27 %), clinical nurses with diploma (66 %) and health assistants (2.8 %). The median age of the respondents was 26.0 years and females made up of 45 %. Generally the knowledge and skills of the respondents were found to be poor while attitude towards leprosy was positive for the majority of the respondents. The result showed that 519 (86.3 %) had poor knowledge. Overall 155 (25.8 %) of the respondents had positive attitude towards leprosy while 205 (34.1 %) had intermediate (mixed) attitude and 241 (40.1 %) had negative attitude to the disease. Among 83 respondents assessed for diagnosis of leprosy only 15(18.0 %) diagnosed leprosy correctly. Variation in knowledge and attitude indicated a significant difference ($p < 0.05$) among different health institutions, professions, gender, in-service training and years of experience.

Conclusion: The current finding underlines that although leprosy control activities are integrated to the general health services in the country, the knowledge and skills of leprosy diagnosis, treatment and management by health workers was unsatisfactory. Hence, attention should be given to develop training strategies that can improve health worker knowledge and promote better leprosy management at public health facilities. This could be achieved through preservice and in-service training and giving adequate emphasis to leprosy related practical work and continuous follow-up.

Keywords: Ethiopia, General health workers, Knowledge, Attitude and practice, Leprosy, Leprosy management, Multi-Drug treatment

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